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The effects of stress on the microbial ruminal environment in beef cattle and its relationship to feed efficiency and methane emissions

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Declaration

I declare that this thesis is my own composition, and the research contained within it is my own work, except where acknowledged. The work that has been described in this thesis has not been submitted for any other degree or professional qualification.

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Abstract

There is currently a poor understanding of the impact of chronic stress on the behaviour and physiology of beef cattle. In monogastrics, hormones released in response to stress can have deleterious effects on the balance of the microbiota present in the gut, which can last long after stress hormone levels have returned to normal. If comparable changes occur in the rumen microbiota, these could have important consequences for ruminal fermentation and digestibility, leading to suboptimal use of nutrients and increased methane emissions. However, there is little information regarding the effects of stress on the rumen microbiome. The overall aim of this thesis was to understand how commercially relevant stressors affect ruminal microbial populations, as well as concurrent effects on behaviour, feed efficiency and methane emissions in beef cattle.

The first objective was to assess the direct contribution of glucocorticoids such as cortisol in mediating the effect of stress on the microflora and feed efficiency. Dexamethasone, a potent synthetic cortisol analogue, was used to evaluate the effect of an exogenous glucocorticoid on feed efficiency and the rumen microbial populations. This treatment was applied to 516 ± 50-day old Limousin cross steers selected based on extremes of feed efficiency, to contrast the effects on ruminal microbial communities of more efficient and less efficient animals. Animals in the treatment group (n=24) were injected with 0.05 mg/kg dexamethasone intramuscularly for 3 consecutive days, while matching extremes of feed efficiency controls (n=16) were treated with the equivalent volume of saline solution (Control group). The effect of dexamethasone was assessed on faecal cortisol metabolites, feeding behaviour, locomotor activity, as well as metagenomic information obtained

from 16S rRNA gene sequencing of rumen fluid samples. Treatment with exogenous glucocorticoid dexamethasone induced transient changes in activity and faecal cortisol. Nonetheless, this glucocorticoid did not cause any significant changes in the rumen archaea population or microbial diversity, an indication that ruminal microbial populations might be resilient to the direct effects of exogenous glucocorticoids.

The second objective was to quantify the behavioural and physiological responses to a putative composite chronic stressor treatment, by applying a series of commercially relevant stressors and assessing any changes in behaviour and HPA axis responsiveness. The commercially relevant stressors used were reduced space allowance, in addition to being subjected every week to regrouping, transport and a short period of isolation. Limousin (n=32) and Angus (n=32) crossbred beef steers, 400 ±13 days old at the beginning of the trial, were assigned in a balanced way to a composite stressor (S) or control (C) treatment, each treatment with four replicate groups. Blood samples and faecal samples were collected to measure cortisol levels. An ACTH challenge was performed using 0.5ug/kg of Synacthen Depot® with blood samples taken just before, 30 min post and 60 min post ACTH challenge to assess changes in HPA axis responsiveness on a subsample of animals (S=22; C=19). Behaviour was assessed from activity monitors, feed intake, an attention bias test, video observations of agonistic behaviour at the feeders and affiliative behaviour (rubbing and licking) in the home pen. Results showed differences between treatments in some parameters of activity and an attention bias test. However, there were no effects of treatment on agonistic or affiliative behaviour. Cortisol responses, although different between groups, could not be specifically attributed to the composite stressor treatment and the ACTH challenge employed did not detect any significant differences in adrenal sensitivity between treatments.

Making use of metagenomic information obtained from 16S rRNA gene sequencing of rumen fluid samples taken during the composite stressor experiment, the last objective of this project assessed changes in the rumen microbiota in response to the applied stressors. Although there was a particular interest in the methanogenic populations, analyses also looked at effects on productivity and methane emissions. To this end a small cohort of 12 animals (six from each C and S treatments) was used to assess any effects of the composite stressor treatment on methane emissions. Although changes were detected in some microbial genera throughout the experiment, there were no major changes in the rumen archaea population or microbial diversity directly associated with the composite stressor treatment. Additionally, by the end of the experiment, there was no effect of stress on growth performance or methane emissions.

In conclusion, circulating glucocorticoids do not appear to affect the rumen microbiota balance directly, although it is possible that they may affect microbial communities in other sites of the gastrointestinal tract. Some differences in behaviour but not cortisol were found in response to the composite stressor treatment, suggesting that beef cattle might be resilient to repeated but predictable stressors. The stressor regime applied did not cause substantial changes in rumen microbial diversity or methanogenic archaea populations. This thesis was a first step towards enhancing our understanding of the dynamics of the rumen microbiome in response to stress. Further research needs to examine more closely the links between biological changes in response to severe chronic stress and microbiota resilience in the rumen and other sites of the gastrointestinal tract.

Lay summary

Cattle can digest plant-based foods such as grass that humans or other animals are not able to digest. This ability to break down fibrous foods into more digestible sub-products is due to microbes which are normal inhabitants of their forestomach, referred to as the rumen. Beef cattle rely heavily on these rumen microbes for their nutrition and growth; therefore, any harmful changes in these microbial communities can lead to reduced productivity and less efficient production of meat. Additionally, as these microbes are responsible for greenhouse gas emissions from cattle, changes in the balance of the rumen microbial community could affect carbon emissions from the animal.

There is limited information available on the effects that stress can have on the rumen microbes. My research tries to understand how stress can affect these microbes in the rumen. To do this, I set up two experiments. In the first experiment, I studied the response of the rumen microbial community to a synthetic version of cortisol; a hormone that mediates the animal's response to stress. The results did not reveal any substantial effects of the drug on the rumen microbes, which could indicate that they are resistant to change in response to these hormones.

The second experiment evaluated whether stress resulting from a mixture of four common farm practices affected behaviour, levels of stress hormones and microbial populations. The stressors applied were a reduction in space per animal, being mixed with new animals, transported and separated from other animals for a short period. The animals showed changes in some of the behaviours evaluated due to the stressors applied, but no changes in blood cortisol levels. There were no changes in the growth performance of the cattle, nor in the rumen microbes.

In conclusion, a synthetic version of the hormone cortisol did not affect the rumen microbes. It also appears that the combination of farm practices used to stress the animals was not enough to induce clear signs of chronic stress, meaning that cattle may have adapted to these stresses because they were predictable and not severe. The rumen microbial communities appear to be resistant to common stressors encountered in commercial production, but more research is necessary to study stress effects on other parts of the digestive system.

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It takes a village…

All the work developed throughout my PhD journey involved the help of a large group of people. It definitely takes a village to do a complex research project, and I would like to dedicate this work to all of those who helped me make this project a reality.

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List of figures and tables

Abbreviations

AAx	Aberdeen Angus crossbred steers
ACTH	Adrenocorticotropic hormone
ANCOM	Analysis of Composition of Microbiomes
ANS	Autonomic nervous system
ASV	Amplicon sequence variants
CH_4	Methane
clr	Centred log-ratio transformation
CMS	Chronic mild stress
CNS	Central nervous system
CO_2	Carbon dioxide
CRH	Corticotropin-releasing hormone
CS	Crush Score
CV	Coefficient of variation
DADA	Divisive amplicon denoising algorithm
DEX	Dexamethasone treatment
DLWG	Daily live weight gain
DMI	Dry matter intake
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric Nervous System
FCR	Feed conversion ratio
FS	Flight Speed
GI	Gastrointestinal
H_2	Hydrogen
HPA axis	Hypothalamic-pituitary-adrenal axis

IQR	Interquartile range
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis effect size
LIMx	Limousin crossbred steers
LMM	Linear mixed models
NGS	Next-Generation Sequencing
NMDS plots	Non-linear multidimensional scaling plots
nts	Nucleotides
OTU	Operational Taxonomic Units
PBS	Phosphate-buffered saline
PLS-DA	Partial Least Squares Discriminant Analysis
RFI	Residual feed intake
SAM axis	Sympathetic-adrenal-medullary axis
TBE	Tris-Borate EDTA
VFAs	Volatile fatty acids

Contents

Chapter 1 - General introduction

1.1 Introduction

The ability of cattle to turn fibrous feedstuff not fit for human consumption into milk and meat has made this species one of the most important in global food supply chains (Morgavi et al., 2010). Ruminants are able to convert energy from cellulose found in fibrous plant matter thanks to a diverse microbial community inhabiting the rumen, composed of bacteria, protozoa, fungi, archaea and viruses living in a symbiotic relationship with their ruminant host. These microbial communities are often referred to collectively as the rumen microbiota, or rumen microbiome when referring to the microbial genome found in the rumen. This complex network of microorganisms is essential to the biology of its ruminant host. As such the disruption of the normal microbiota balance can have a substantial effect on the health, welfare and overall production efficiency of cattle (Jami and Mizrahi, 2012; Henderson et al., 2015; Malmuthuge et al., 2015).

The need to increase productivity has made the beef cattle sector adopt more intensive production systems that deviate from the environment to which cattle are adapted. Therefore, a consideration of animal welfare has been included in livestock production systems in efforts to balance productivity with the ethical concern and needs of the animal. Animal Welfare Science deals with understanding and assessing the effects of the conditions in which animals are kept on the physical and mental state of animals under our care. Due to its relationship to both the physical and mental state of an animal, the concept of stress is strongly interlinked with animal welfare, mainly because many welfare problems will cause stress (Fraser et al., 1975; Villalba and Manteca, 2019). Not all stressors will necessarily affect animal welfare, as

stressors are a part of every environment and therefore unavoidable. As such, responding to stressors in order to maintain homeostasis is adaptive. However, where stress is perceived as aversive or to constitute suffering, animal welfare will be compromised.

The general concept that stress can lead to negative impacts on the health, welfare and productivity of animals is widely accepted (Burdick et al., 2011a). Stress can affect key physiological processes. Consequently, prolonged stress has been found to increase catabolism, affect immunity, and lead to deleterious effects on metabolism, growth, and reproduction (Blecha, 2000; Elsasser et al., 2000, Burdick et al., 2011a). Together with the psychological effect of stress, these potentially unfavourable effects of stress can pose a threat to cattle health and welfare, as well as having important implications from a productive and an economic point of view. The rumen microbiome is a highly complex biological system and could play a major role in how stress impacts metabolism and production. Despite this, the effects of stress on the rumen microbiome of cattle are still not well understood.

The following literature review will address the current gaps in understanding of the effects of stress on the ruminal microbial environment and its homeostasis. The first section of this chapter provides a general overview of the stress response, as well as describing how acute and chronic stress can affect biological function when demands surpass capacity to cope with stress. The subsequent section will give a basic introduction to the rumen microbiome and metagenomic analyses, discussing some of the putative links between stress and productivity and methane emissions in beef cattle. Finally, literature exploring the links between the microbiome and behaviour and stress responses in other species will be explored to identify knowledge gaps in regards to the effects of stress on the rumen microbiome. Lastly, the research questions this thesis addresses and chapter structure used will be briefly described.

1.2 The biology of stress

Stressors are internal or external factors that threaten to disrupt an organism's homeostasis, triggering a stress response (Wiepkema and Koolhaas, 1993). What an animal will perceive as a stressor is highly subjective. Triggering of a stress response does not necessarily require that the stressor poses an actual threat to homeostasis, but rather that it is perceived as a potential danger. Often a stress response is entirely anticipatory to a stimulus that could potentially be a threat and is frequently influenced by previous experience (Hernández-Cruz et al., 2016). A state of stress can be defined as an abnormal or extreme adjustment in the physiology of an animal to cope with a stressor (Fraser, 1975).

Mechanisms to cope with stressors entail activation of cascades of events to respond both in the short term to the immediate threat, as well as in the long term, to accommodate the increased demand on body reserves. Therefore, it is through this process that the animal can maintain allostasis in response to an environmental challenge. These mechanisms involve autonomic nervous system (ANS) activation of the sympathetic-adrenal-medullary (SAM) axis. The SAM axis causes the release of catecholamines (e.g. adrenaline and noradrenaline) by the adrenal medulla and sympathetic nerves (Kvetnansky et al., 2009; Tank and Lee Wong, 2014), for an immediate response aimed at enabling sudden and sustained physical activity, commonly referred to as Fight or Flight response. Additionally, a neuroendocrine response by the hypothalamic-pituitary-adrenal (HPA) axis is also activated, inducing a release of glucocorticoids (e.g. cortisol) which have critical functions for coping with stress such as sustained catabolism and anti-inflammatory effects (Joëls and Baram, 2009; Tank and Lee Wong, 2014). Specifically, this stress response to internal or external stressors involves the central nervous system activating the HPA axis (see Figure 1.1), where an increased synthesis of corticotropin-releasing hormone (CRH)

from the paraventricular nucleus of the hypothalamus is secreted into portal circulation within a few seconds (Gupta et al., 2004; O'Connor et al., 2000). Once CRH reaches the anterior pituitary, a group of cells called corticotrophs secrete adrenocorticotropic hormone (ACTH) (Gupta et al., 2004; Mormède et al., 2007). ACTH then travels through the general circulation to the adrenal glands, where it causes the release of glucocorticoids (cortisol or corticosterone) from the adrenal cortex (Romero and Butler, 2007). Cortisol is the main glucocorticoid in cattle, and it has a critical function in regulating energy homeostasis during stress responses, thereby preparing the body for exertion, in addition to having anti-inflammatory and immunomodulatory effects (Moberg, 2000). In experimental settings, the increased levels of glucocorticoids or their metabolites in blood or other tissues is commonly used to characterise the stress response (Cockrem, 2013). Glucocorticoids are also involved in mediating and modulating the overall stress response, exerting a negative feedback on the hypothalamus and pituitary, hence being able to alter the release of CRH and ACTH, affecting the magnitude and duration of the endocrine response to stress. Hence, under normal circumstances, this negative feedback limits exposure to high levels of cortisol (Moberg, 2000). Both glucocorticoid and mineralocorticoid receptors are involved in this negative feedback of cortisol in stress responses, which is also crucial for the normal circadian pattern of cortisol secretion (Otte et al., 2003; Berardelli et al., 2013).

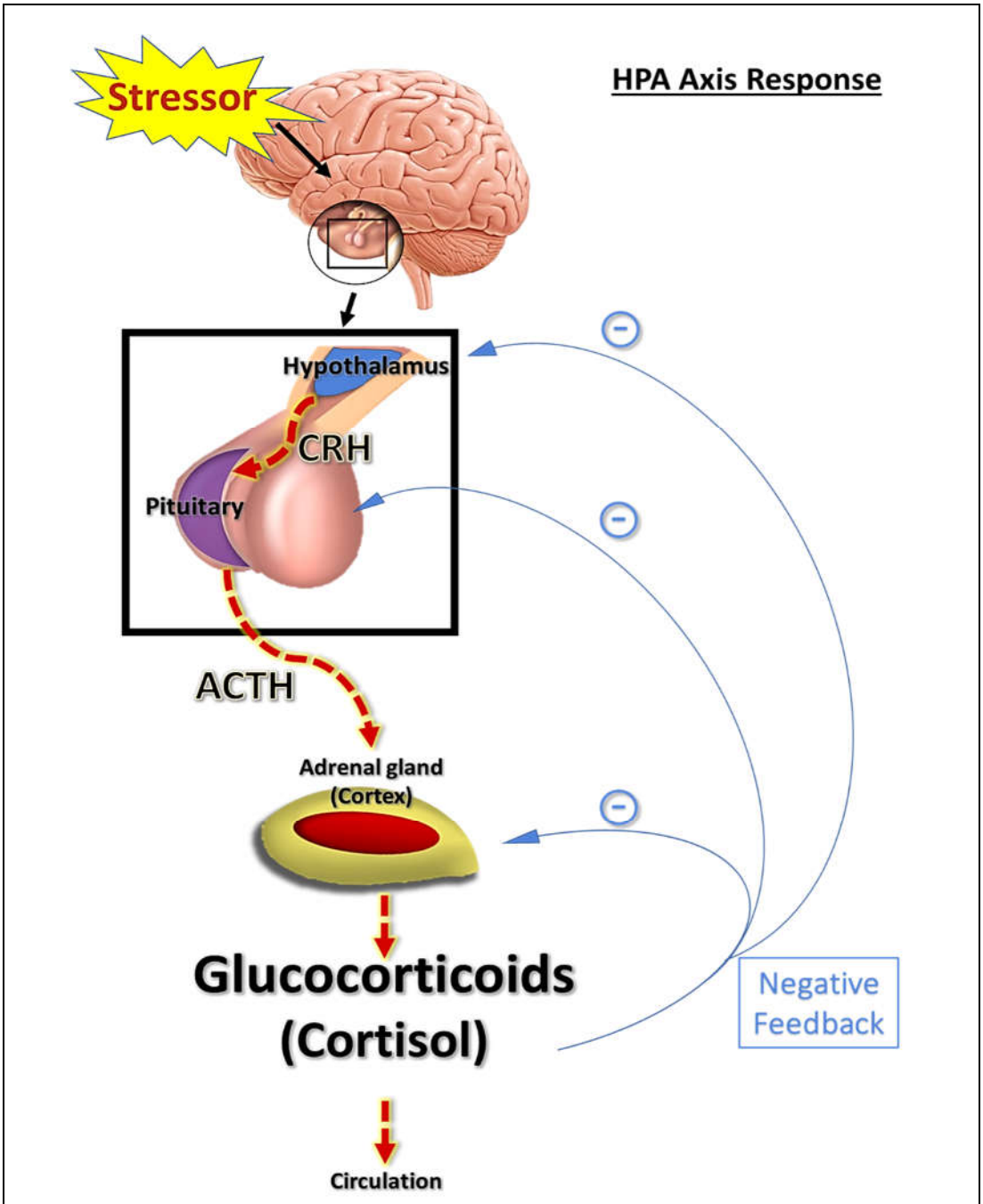


Figure 1.1. Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis response and negative feedback loop for glucocorticoid (e.g. cortisol) production.

The response to stressors, besides neuroendocrine and ANS responses, may involve a temporary shift in behaviour to cope with, avoid or remove the threat. For example, an animal exposed to hot weather will likely search for shade or shelter when heat

threatens to affect homeostasis. Behavioural responses are the most economical and most frequently used strategies to recover homeostasis. Behavioural responses tend to be short in duration and commonly work in conjunction with physiological strategies. This is evident in Fight or Flight responses which involve a clear physiological and behavioural response to remove or escape the stressor. Individual variation in behavioural responses to stressors occurs, guided by previous experience with the stressor as well as individual factors such as temperament. Some of these behavioural changes can also be used as indicators of stress (Grandin, 1997; Haley et al., 2000; Barrell, 2019). However, it is important to note that the opportunity to perform adaptive behavioural strategies may be limited in commercial confinement environments, preventing the animal from implementing the behavioural choice necessary to mitigate the stressor. This has implications for the thwarting of motivations, capacity to cope and the likelihood of further stress (Moberg, 2000).

According to its duration or temporal exposure, stress has traditionally been classified as acute or chronic (Moberg, 2000; Trevisi and Bertoni, 2009). Acute stress usually refers to a brief exposure to a single stressor. Acute stress responses facilitate and promote a quick adaptation in the short term. Therefore, recovery from acute stress tends to happen relatively swiftly, enabling the return of the physiological balance. Thus, these responses tend to be adaptive. Normal acute stress responses are sensitive to intrinsic feedback mechanisms restraining over-reaction from the central and peripheral components of the stress system, as the catabolic and immunosuppressive effects of the acute stress response are intended to be beneficial to the individual over a limited duration of time (Hughes et al., 2014). However, even acute stressors can still cause disturbances if severe enough to alter biological function or mental state (Moberg, 2000; Trevisi and Bertoni, 2009).

On the other hand, chronic stress refers to more prolonged stress resulting from a long duration stressor or series of acute stressors. This may result in a prolonged insult to the homeostatic state and persistent exposure to a high level of glucocorticoids. This can incur a high biological cost in the form of distress and a prepathological or pathological state (Moberg, 2000; Burdick et al., 2011a). The term pathology in this context is not to be confused with its use in the context of disease. Instead, it refers to the abnormal functioning of biological systems, leading to the inability to perform normal biological functions, and is manifest through impairments to reproduction, reduced growth or normal behaviour (behavioural pathology). For the development of pathological conditions, the shift towards chronic stress is dependent upon the duration and severity of the stressor, the animal and its perception of the stressor, together with its ability to cope with the stress, previous exposure, genetics, temperament and other contributing factors (Hughes et al., 2014). The accumulation of biological costs can be the result of repetition of the same acute stressor or due to the combined effect of different stressors (Moberg, 2000). Responses to stress are dynamic. In response to repeated exposure to a stressor, stress responses may decrease over time by modulation at multiple levels or habituation in the long term, causing a decrease in activation of the HPA axis (Lay et al., 1996; Mormède et al., 2007).

On the other hand, chronic stressor responses may show changes in sensitivity in an attempt to control the deleterious effects of HPA axis activation in response to an unavoidable stressor. For example, the increase in cortisol after the onset of a stressor may be diminished later by changes due to the intrinsic feedback mechanisms that prevent extended exposure to high glucocorticoid concentrations, showing dampened cortisol response to the stressor over time (Trevisi and Bertoni, 2009; Reiche et al., 2020)

Some authors describe that from a physiological standpoint, the defining feature of the stress response is the activation of the HPA axis (Gupta et al., 2004). Although the activation of the SAM and HPA axis is a direct consequence of the physiological responses occurring due to a stressor, some situations that can induce this neuroendocrine activation are not events we would generally class as stressors or as having a negative valence for the animal. For example, HPA axis activation can occur in response to exercise, sexual behaviour and nursing behaviour (Rushen and Passillé, 1992), showing increased glucocorticoid responses in situations that typically would not be classed as stressful. Hence, Broom and Johnson (2019) argue that as situations with a positive valence for the animal can also activate HPA axis responses, just to equate stress to activation of the HPA axis would be inadequate. Therefore, HPA activity alone does not elucidate the point at which a stressor becomes deleterious for the animal.

A useful framework to conceptualise the processes involved in a stress response is described by Moberg (2000), where a stress response can be considered as divided into three general stages (see Figure 1.2). The first stage involves the recognition of the stressor by the Central Nervous System (CNS), triggering the second stage where a biological response is mounted. The last stage describes the biological consequences of stress. Here the changes in biological function to cope with stress and the degree of shift in biological resources away from other basic biological functions (such as growth or reproduction), which could be viewed as "the biological cost of stress", will dictate whether the biological cost is negligible to the normal functioning of the body or if welfare is compromised. Broom and Johnson (2019) describe a maladaptive response to stress as an environmental effect that overtaxes the animal’s control system, resulting in adverse consequences and eventually reduced fitness. Chronic stress may lead to distress and pathological states due to

insufficient reserves to deal with the stress response or subsequent stressors (accumulated biological cost of stress). However, this pre-pathological or pathological state will last until the animal can replenish biological resources sufficiently to restore normal functioning, can remove itself from the stressor or alter the perception of the stressor. Therefore, this process can be altered at multiple levels.

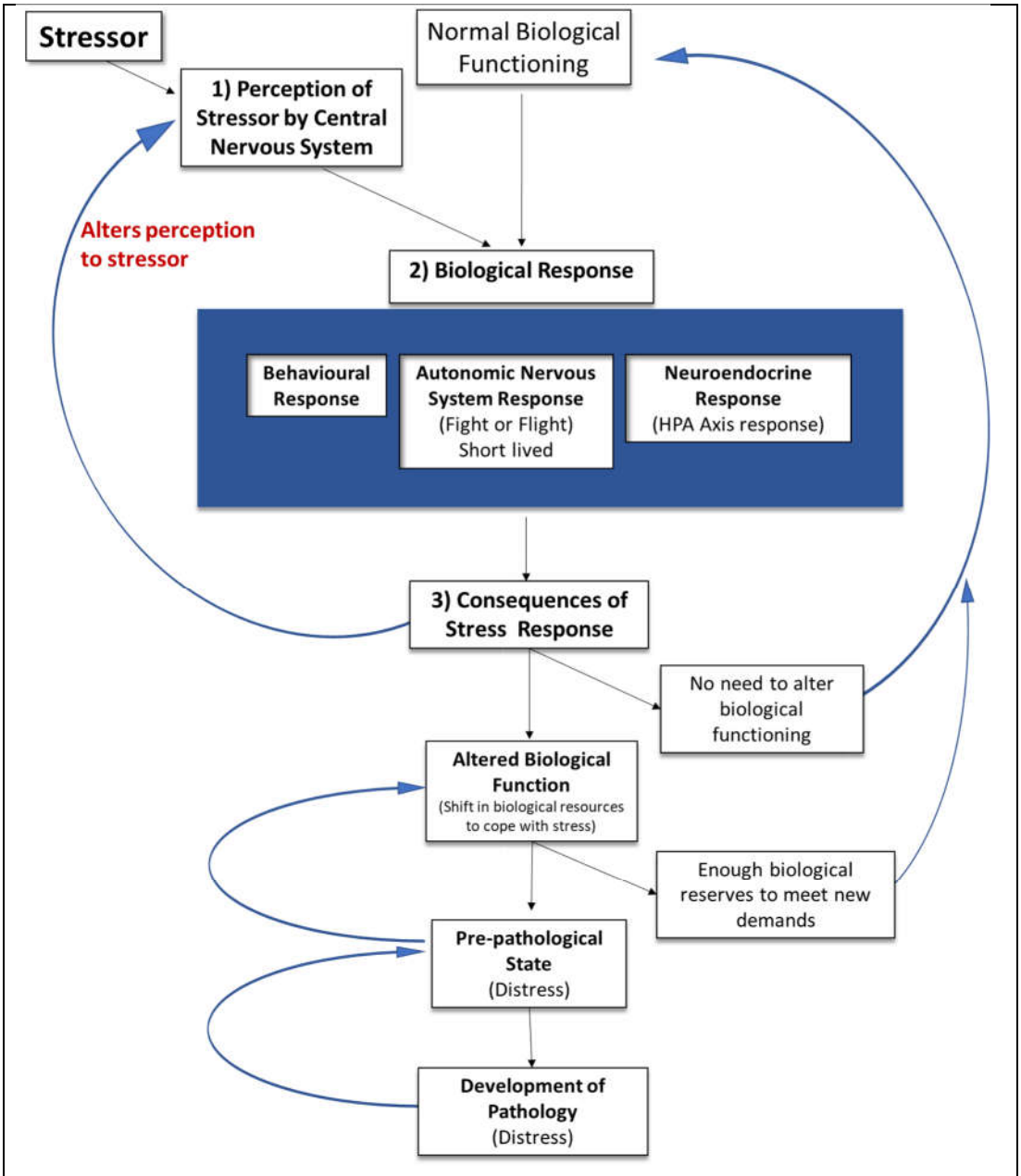


Figure 1.2. Visual representation of a model to describe the biological response to stress. Adapted from framework described by Moberg (2000).

Given the essential function of rumen microbial communities to the biology of cattle, some of the effects of acute and chronic stress in ruminants could be partly mediated by a disruption in the normal rumen microbiome of the animal. The subsequent section of this chapter introduces the reader to metagenomic analyses in order to discuss evidence exploring the links between the microbiome with behaviour and stress responses in other species, and to identify relevant unanswered questions on the effects of stress on the rumen microbiome.

1.3 The Microbiome

As mentioned previously, whilst the microbiota refers to the microorganisms present in a particular site or environment, the microbiome can be defined as the collection of the genetic material of microbes in a specific environment (Turnbaugh et al., 2007). The advent of Next Generation Sequencing techniques has facilitated the detection of sequences related to many different microbes in a sample without having to rely on culturing microorganisms. The arrival of more accurate and cheaper sequencing technologies in recent years is one of the drivers for the rapid increase in microbiome research, with a particular focus on the types of microorganisms that live in a healthy body and changes in these communities under pathological conditions.

1.3.1 Metagenomics

Metagenomics is the study of genetic material (microbial genomes) of a complex mixture of microorganisms retrieved from an environment (Wooley et al., 2010; Oulas et al., 2015). The rumen contains a very complex and diverse population of microorganisms, most of which are obligate anaerobes. This makes their identification by classic culture and microscopy methods complicated, and quantification nearly impossible by traditional methods. The advent of culture-independent methods using the 16S rRNA gene and subsequent development of Next Generation Sequencing (NGS) has brought tremendous insight into the study of microbial populations and their dynamics. NGS 16S rRNA gene sequencing takes advantage of the importance of ribosomes in cells of living organisms which play a key role in protein synthesis. In prokaryotes, the 16S rRNA gene is around 1500 bp in length, and it is highly conserved due to its essential role in the expression of the 16S ribosomal subunit which is vital in mRNA translation and protein synthesis. Therefore, the sections of this gene that are essential for expressing 16S rRNA are very conserved and show minimal mutation rates, whereas sections that are not essential for this function show

a higher variability between species (Liu et al., 2007). This characteristic leads to conserved regions common to all prokaryotes, next to hypervariable regions that change between species. This makes the 16S rRNA gene a popular target to identify bacterial and archaeal communities (see Figure 1.3).

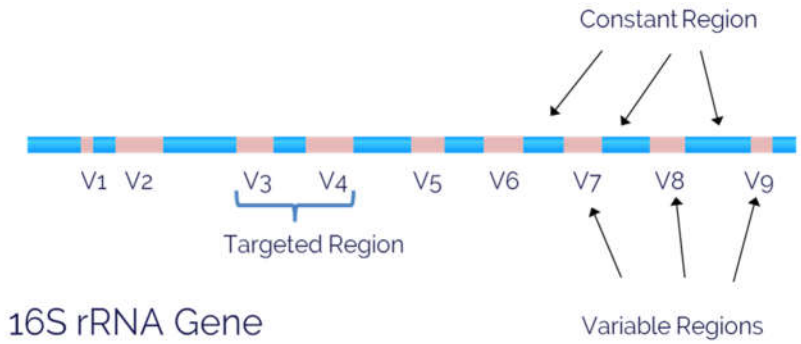


Figure 1.3. Schematic diagram of the 16S rRNA gene, showing conserved regions in blue and variable regions in pink.
Modified from https://www.lcsciences.com/discovery/applications/genomics/16s-mobile/

Sequencing the full 16S rRNA gene is still costly and time-consuming, especially when dealing with a large number of samples. As such, techniques have been developed where only a part of the 16S rRNA gene is sequenced, using portions of the conserved regions as a guide to identifying the sequences that belong to a variable region (Chakravorty et al., 2007). In addition, metabarcoding can be used to identify individual samples, allowing for many samples to be sequenced within the same run by multiplexing. This barcode information can be filtered bioinformatically (i.e. demultiplexing) to identify the sequences derived from each sample (see Figure 1.4). For example, in the Illumina MiSeq and HiSeq platforms, the DNA is extracted from the rumen environment and broken into small fragments. Primers are used to match the conserved region that immediately precedes and follows the variable region of interest. This makes it possible to amplify and sequence only those sections of the DNA with a higher change rate between microbial groups to enable taxonomic identification (Chakravorty et al., 2007).

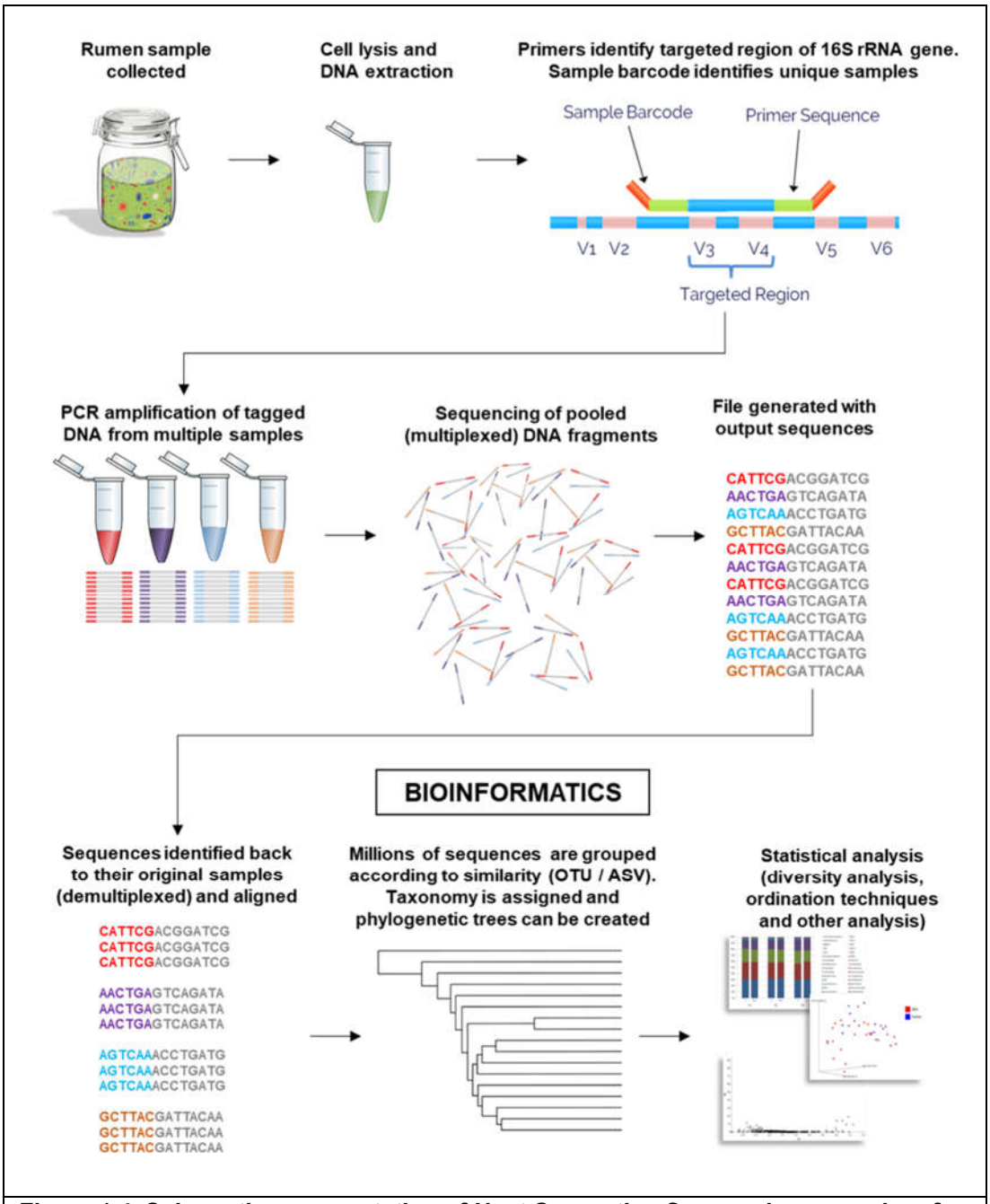


Figure 1.4. Schematic representation of Next Generation Sequencing procedure for metagenomics using 16S rRNA gene sequencing. Adapted from Caporaso et al., 2010 and Hamady et al., 2008.
Third image modified from https://www.lcsciences.com/discovery/applications/genomics/16s-mobile/

For further analysis, sequences are usually then grouped according to their similarity. Typically, this is done by clustering sequences that differ by less than a fixed threshold, commonly less than 3% sequence dissimilarity in the case of grouping by Operational Taxonomic Units (OTU) (Westcott and Schloss, 2015; Kopylova et al.,

2016;). More recently, grouping by amplicon sequence variants (ASV) has been suggested. This method uses model-based approaches to cluster inferred biological sequence variants within a sample, distinguishing variants differing by as little as one nucleotide (Eren et al., 2013; Edgar and Flyvbjerg, 2015; Callahan et al., 2017).

ASV analysis is different in the sense that instead of grouping using a similarity threshold, such as the 97% similarity used in OTU clustering to determine a consensus sequence, it uses the exact sequence of nucleotides (i.e. which exact sequences were read and how many times they were read). An error model is subsequently run to determine the probability that a given read at a given frequency is not due to sequencing error. After filtering, this leaves exact sequences that had a given level of statistical confidence. As these are exact sequences, they can be compared between studies using the same target region, and in theory can be associated to a reference database at a much higher resolution (a read sequence is compared to a reference sequence, rather than a consensus cluster being compared to a reference cluster or read).

ASVs can then be computationally identified to taxonomy using reference databases such as Greengenes or SILVA (DeSantis et al., 2006; Quast et al., 2012). This taxonomic information contains not only the microbial groups present but can also be used to calculate the relative abundance of taxonomies within the sample. These relative abundances of taxonomies can then be used to compare community structures using metrics that quantify diversity. They also allow the use of more complicated bioinformatic tools to compare changes in individual taxonomies associated with different categorical variables, for example to compare differences between groups of animals or sampling locations within the host.

1.3.2 The bovine rumen microbiome

The rumen microbiome has evolved with ruminants over time, facilitating the degradation of fibrous plant matter that otherwise would not be digestible by its host. In turn, natural selection has turned the rumen into a specialised large volume anaerobic fermentation chamber containing a complex microbial ecosystem. Bacteria are the predominant microbial group in the rumen, followed by archaea. Protozoa, although less in number than archaea, can make up to half of the rumen biomass due to their large size, and lastly, fungi are much fewer in number (Lourenço et al., 2010; Kumar et al., 2013; Wang et al., 2017). Ingested plant biomass is degraded by rumen microbiota producing volatile fatty acids (VFAs) during hydrolysis and fermentation, as well as ammonia, microbial protein, and vitamins, among other compounds (Krehbiel, 2014). This ecosystem and symbiotic relationship with the host is so specialised that VFAs produced by the microbiota can meet up to 70% of the daily energy requirements of the host (Bergman, 1990) functioning as the primary source of energy for the animal (Söllinger et al., 2018). This highlights the importance of a well-functioning microbiome for the biology of ruminants.

An important secondary product of anaerobic fermentation in the rumen is the production of carbon dioxide (CO_2) and hydrogen (H_2). One of the necessary conditions for the normal functioning of the rumen is a pH between 5.5 and 7.2 and levels below these values can induce serious metabolic consequences (Plaizier et al., 2008). Methanogenesis is one of the main processes by which the inhibition of fermentation by a low pH is avoided by converting excess H_2 and a carbon substrate (commonly CO_2) to methane (CH_4). This process is performed by methanogenic archaea. Methane emissions are considered one of the foremost anthropogenic contributors to the greenhouse effect (Opio et al., 2013). It has been calculated that rumen methanogens are responsible for around 20% of global methane emissions

(Hua et al., 2018). Methane also has a high global warming potential, around 23 times that of CO_2, with a shorter atmospheric lifetime (Crosson, 2008; Asner and Archer, 2010). Therefore, mitigating methane emissions could have a more immediate impact on the reduction of the greenhouse effect than other gases. There are several strategies currently being evaluated to reduce ruminant methane emissions (Eckard et al., 2010; Gerber et al., 2013a; Herrero et al., 2016) and it has been suggested that individual variation in host responses to stress could underlie part of the variation in methane emissions in livestock (Llonch et al. 2016). Therefore, understanding the role played by stress on the microbiome, and especially methanogenic communities, is potentially of great importance for methane mitigation strategies.

1.4 Microbiome and the "second brain" – links between stress, behaviour and the gut microbiome

Due to the vast number of synapses and neurones that comprise it, the Enteric Nervous System (ENS) is commonly referred to as the "Second Brain" (Gershon, 1999). In humans, over 500 million neurons comprise the ENS (Furness and Costa, 1987) and it is now increasingly recognised as a separate division of the Autonomic Nervous System different from the Sympathetic and Parasympathetic branches, as the ENS has its own independent reflex activity. The importance of this brain-gut axis has been increasingly studied over recent years, and it has been seen that an appropriate microbiota may play an essential role in this brain-gut axis balance (O'Mahony et al., 2009; Dinan and Cryan, 2012). Therefore, bidirectional communication appears to exist in this microbiome-gut-brain axis, where the animal can influence its microbiota composition and motility via feeding, individual genetics, physiology and nervous system interactions. At the same time, the microbiota can act directly or indirectly on the brain via the release of structural components (such as lipopolysaccharides) or metabolites (e.g. neurotransmitters, catecholamines, indole) that can activate proinflammatory pathways, stimulate the Enteric Nervous System or induce secretion of neuropeptides (Kelly et al., 2016; Sharon et al., 2016; Kraimi et al., 2019; Sgritta et al., 2019). For example, it has been found that germ-free mice are deficient in serotonin, and that the addition of specific microorganisms and microbial metabolites can increase serotonin production (Yano et al., 2015). Similarly, it has been found that the exchange of the microbiome between anxious and non-anxious mouse phenotypes induces a change in those behavioural traits in the host. Therefore, a microbiome transplant from an anxious mouse produces an anxious phenotype, and that of a non-anxious mouse produces a non-anxious phenotype (Collins et al., 2013). Also, it has been found that the addition of certain probiotic

bacteria can modulate the GABAergic system in mice, and if the vagus nerve is severed, this mood-modulating effect is lost (Bravo et al., 2012) suggesting that there is some effect of the microbiota on mood and behaviour.

Microbial endocrinology is a discipline that hypothesises that through their long coexistence with animals and plants, certain microorganisms have evolved systems to detect chemicals from the host such as hormones. The presence of these chemicals could signal to the microbe that the environment is suitable for its replication, such as the expression of genes involved in colonisation or virulence in pathogens (Freestone, 2013). For example, it has been found that the addition of stress hormones such as catecholamines to a growth medium can induce exponential growth in colonies of *Escherichia coli*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* (Lyte and Ernst, 1992). Similarly, other bacteria have been found to react to further hormone types. For example, *Burkholderia pseudomallei* has a specific and high-affinity binding site for insulin (Woods et al., 1993), which might explain why diabetes mellitus is the most significant risk factor for developing Melioidosis, which is the disease resulting from infection with *Burkholderia pseudomallei* (Hassan et al., 2010). Therefore, given that microbes can respond to endocrine signalling, the evidence seems to suggest that certain microbes that proliferate following a stress event might not necessarily take advantage of a reduction in immune function, but may instead directly respond to the presence of stress hormones or other stress-related metabolites.

1.4.1 Stress implications for the rumen microbiome

Previous research has shown that hormones released in response to stress can have deleterious effects on the balance of the microbiota present in the gut which can last long after stress hormone levels have returned to normal (Freestone and Lyte, 2010).

However, much of the research in this area is in monogastrics. Therefore its translation to the rumen microbiome is limited, and research evaluating the effects of stress on the rumen is scarce. Changes in the normal rumen microbiome may have important consequences for ruminal fermentation and digestibility, leading to suboptimal use of nutrients and increased methane emissions.

In cattle, a few studies have found changes in rumen microbial populations in response to heat stress (Tajima et al., 2007; Uyeno et al., 2010; Chen et al., 2018; Zhao et al., 2019; Baek et al., 2020), whilst two other studies have shown transitory changes in the microbiome following short term transport stress in beef cattle (Deng et al., 2017). These results show that there could be changes in the microbiome in response to stress; however, these results are difficult to separate from changes in feed intake. A complementary finding suggested that beef cattle that respond to mild transport stress with a higher cortisol release produced more methane per kilogram of feed consumed when measured several weeks after the imposition of the stressor (Llonch et al., 2016). This novel insight highlights the need to understand the biological mechanisms underlying this relationship and whether the microbiome is involved.

It has also been estimated that protein turnover, tissue metabolism and stress responses can account for 37% of the variation in feed efficiency in beef cattle (Richardson and Herd, 2004), but the precise contribution of stress and the mechanisms driving this are currently unknown. However, we do know that low feed efficiency and higher methane emissions in beef cattle are associated with differences in the absolute abundance and relative proportion of methanogenic populations (Nkrumah et al., 2006; Zhou et al., 2009, 2010). In this regard, a recent study has shown that the relative abundances of some microbial genes in the rumen are

associated with host feed efficiency, growth rate and feed intake in cattle (Lima et al., 2019). Similarly, the abundance of some microbial genes is strongly correlated with the methane emissions of individual animals (Wallace et al., 2015a). Therefore, it is likely that changes in microbiota provoked by stress responses might change the fermentation pattern in the rumen resulting in variations in digestive function and methane emissions. These findings highlight the need to understand the effects of stress on the rumen microbiome and the consequences for productivity and methane emissions.

1.5 Thesis Outline and Objectives

As discussed throughout this chapter (**Chapter 1**), there is limited information available regarding the effect stressors have on the rumen microbiome. Additionally, the difficulty of separating changes in feed intake from other effects of stress complicates the interpretation of the few studies available. In the case of beef cattle production, we know very little about whether repeated commercially relevant stressors affect the microbiome. My research aims to contribute knowledge to better understand how stress may impact ruminal microbial populations, and concurrent effects of these changes on feeding behaviour, feed efficiency and methane emissions in beef cattle.

The first objective was to assess the direct contribution of glucocorticoids such as cortisol in mediating the effect of stress on the microbiota and feed efficiency. **Chapter 2** presents the results of a trial developed to evaluate the impacts of a circulating exogenous glucocorticoid (dexamethasone) on the rumen microbiome of steers differing in feed efficiency, in order to contrast the effects on ruminal microbial communities of more efficient and less efficient animals. Identifying the direct effects of circulating glucocorticoids will help determine the role that the hypothalamic-pituitary-adrenal (HPA) axis plays in changes in the rumen microbiome. The use of an exogenous glucocorticoid allowed examination of the effect of glucocorticoids on the microbiome in a controlled manner, whilst minimising other behavioural effects, such as changes in feeding patterns. The hypothesis was that repeated injection of a circulating exogenous glucocorticoid (dexamethasone) would alter the composition of the ruminal microbial populations.

The second objective of the thesis was to quantify the behavioural and physiological responses to a composite stressor treatment by applying a series of commercially

relevant stressors and assessing any changes in behaviour and HPA axis responsiveness. **Chapter 3** covers the description of the experimental design and quantification of behavioural and physiological effects of a regime comprised of four commercially relevant stressors (reduced space allowance, in addition to being subjected every week to regrouping, transport and a short period of isolation). The working hypothesis was that the composite stressor treatment applied would alter the behavioural responses, basal cortisol levels and affect stress responsiveness of the animals in this study.

The last objectives of this project were to assess changes in the rumen microbiota in response to the applied composite stressor treatment of Chapter 3, with a particular interest on the methanogenic populations, as well as to analyse any effects on productivity and methane emissions. In **Chapter 4,** the effects of the composite stressor treatment on the rumen microbiome and productivity are described, as well as data from a pilot study to assess any effects of the composite stressor treatment on methane emissions. The hypothesis of this part of the study was that the commercially relevant composite stressor treatment applied would lead to changes in the rumen microbiota, as well as changes in microbial communities responsible for methane production in beef cattle.

Finally, **Chapter 5** presents a general discussion of the main findings of this project, implications and some ideas for future research work.

Chapter 2 - The effects of a circulating glucocorticoid on the rumen microbiome of beef cattle with diverging feed efficiency

2.1 Introduction

Stress responses generate cascades of changes to metabolism, and one of the routes for these changes is through the activation of the hypothalamic-pituitary-adrenal (HPA) axis to produce glucocorticoids such as cortisol, to prepare the body for exertion and to mediate and modulate the overall stress response. Endogenous glucocorticoids play an active role in the mobilization of glucose (Dallman et al., 1993), amino acids (Simmons et al., 1984) and free fatty acids (Xu et al., 2009) from body stores, and influence immune system function (Sapolsky et al., 2000). Prolonged stress and presence of glucocorticoids has been linked to impaired immunity, increased catabolism and deleterious effects on metabolism (Blecha, 2000; Elsasser et al., 2000). This interaction of effects that relate to both immunity and metabolism has sparked an interest in understanding the effect stress and glucocorticoids may have on the normal commensal microbial populations in the gut, commonly referred to as the gut microbiota.

The microbiota serves essential functions as a barrier against colonization by non-normal and pathogenic microorganisms in the gut. It also aids in modulating immunity and produces useful metabolites for the host, such as vitamins and VFAs in the case of ruminants (Bergman, 1990; Freestone, 2013; Blacher et al., 2017). However, previous research in monogastrics has shown that hormones released in response to stress can have deleterious effects on the balance of the microbiota present in the gut which can last long after stress hormone levels have returned to normal (Freestone

and Lyte, 2010). Similarly, some studies in ruminants have found that heat stress in heifers can induce a change in microbial diversity (Tajima et al., 2007; Uyeno et al., 2010). These changes in the normal microbiome may have important consequences for ruminal fermentation and digestibility, leading to suboptimal use of nutrients and affecting productivity.

Although there is some evidence that the enteric microflora is affected by stress, it is still poorly understood whether glucocorticoids such as cortisol are directly responsible for these effects. If this is the case, there exists the possibility that the effect stress has on feed efficiency may be mediated by glucocorticoid hormones affecting microbial populations.

Since ruminants rely heavily on their rumen microbiome to break down cellulose from plant feed, obtain VFA, microbe-derived protein and many other nutrients necessary for their sustenance, we were interested in investigating whether there was evidence of any effects of a synthetic glucocorticoid on the rumen microbiota populations. The glucocorticoid dexamethasone is a synthetic analogue of cortisol, commonly used in veterinary practice to reduce inflammation and the immune response. Dexamethasone has 30 times the potency of cortisol as an anti-inflammatory drug (Papich, 2016), can be recognized by cortisol target receptors and trigger a negative feedback on the production of cortisol (Rayalam et al., 2013). It has been used in the past as a method to probe responses to changes in the HPA axis (Fisher et al., 2002; Raussi et al., 2006; Kelly et al., 2017). Given this functional relationship with cortisol, dexamethasone was used in this experiment to probe the effect of a circulating glucocorticoid on the rumen microbial environment in a controlled manner.

Due to the possibility that the individual animal level of feed efficiency could influence stress effects on ruminal microbial populations, we decided to assess these effects

on cattle of diverging feed efficiency, measured by their Residual Feed Intake (RFI). RFI is the difference between an animal's actual feed intake and the predicted feed intake for its calculated requirements for lean and fat tissue growth as well as its body maintenance requirements. Therefore, RFI indicates whether the animal ate more or less than was predicted for its requirements, and represents feed use efficiency that is independent of mature size effects or level of production (Herd and Arthur, 2009) and is moderately heritable (Berry and Crowley, 2013).

This study therefore aimed to assess the effect of a circulating exogenous glucocorticoid (dexamethasone in this case) on the rumen microbial population and feed efficiency. This dexamethasone treatment was applied to a sub-sample of the animals, selected based on their RFI level, to contrast the effects on the microbial populations of more efficient (low RFI) versus less efficient (high RFI) animals.

2.2 Materials and methods

This experiment was approved by the Animal Experiment Committee of SRUC and was conducted following the requirements of the UK Animals (Scientific Procedures) Act 1986 (PPL 70/8629). The study was carried out at Easter Howgate Farm (Midlothian, UK) from August to December 2016.

2.2.1 Animals and study design

A total of 82 crossbred Limousin steers were used for this study. These animals came from a previous observational experiment to validate intake monitors in a study using two different diets. The steers were 516 ± 50 days old and weighed 531 ± 63 kg at the start of the trial. Animals were divided into four indoor pens of the same design (162 m^2) balanced for weight and sire. Each pen was allocated to either a Concentrate based diet (forage: concentrate ratio of 8:92 on a dry matter basis) or a forage diet (50:50 DM basis). See Appendix 2.1 for diet composition. Food was provided *ad libitum* by eight automatic HOKO single-space bin feeders (Insentec B.V., Marknesse, The Netherlands) in each pen. These feeders provided a daily record of the total dry matter intake (DMI) of each animal. The steers had an adaptation period of 4 weeks to habituate to the diets, home pen and automatic feeders.

Residual feed intake (RFI) was calculated for all animals using 8 weeks of daily DMI data. RFI was calculated as the deviation of actual DMI (kg/d) from DMI predicted based on a linear regression of DMI on average daily live weight gain (DLWG), metabolic body weight at the mid-point of the assessment period and fat depth as per the methods described by Basarab et al. (2003) and Duthie et al. (2016).

Once the individual RFI values were estimated, animals with extreme high and low efficiency in this trait were selected. Only the animals that ranked in the first quartile (with low RFI representing the most efficient) and last quartile (with high RFI indicating

the least efficient) in each pen were selected. The animals remained in their usual home pen along with the animals not taking part in the trial (quartile two and three, which had a medium RFI). See Figure 2.1 for a diagram of the experimental design timeline.

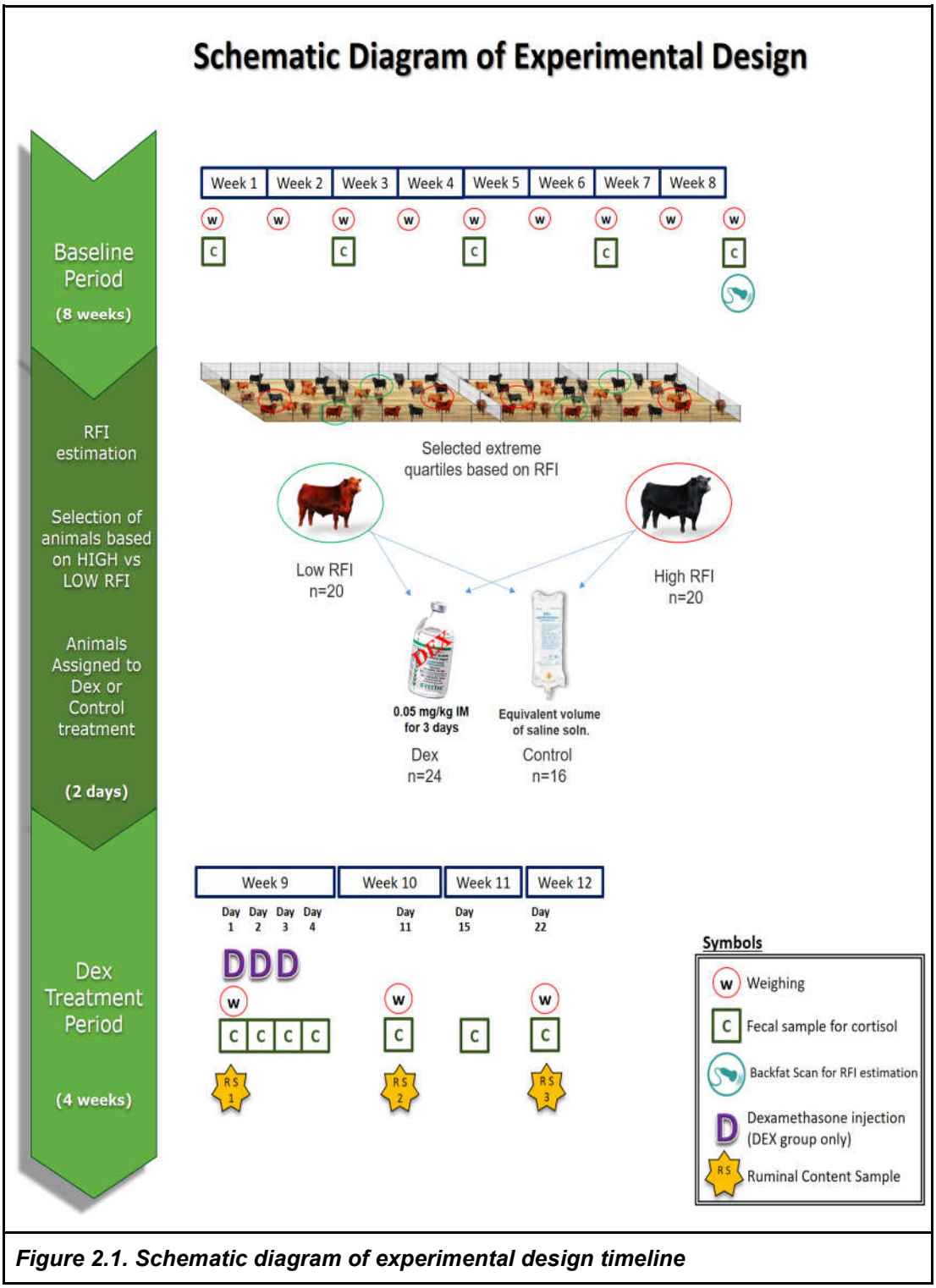


Figure 2.1. Schematic diagram of experimental design timeline

2.2.2 Experimental treatment and study measurements

In each of the four pens, five High RFI and five Low RFI animals were selected (total n=40). For every five animals selected on their RFI, three of these were treated with dexamethasone, and two were used as contemporary controls (exposed to the same handling regime but injected with saline) of the same RFI category. Hence, each pen had three High RFI animals treated with dexamethasone, two High RFI controls, three Low RFI treated with dexamethasone and two Low RFI controls, totalling 24 dexamethasone-treated and 16 control animals in the study. Informed by the methods of Burton and Kehrli (1996), Ilott et al., (1997) and Anderson et al., (1999), animals in the dexamethasone treatment (DEX) group were injected with 0.05 mg/kg IM of dexamethasone (Dexadreson® 2 mg/ml, Intervet, UK) for 3 consecutive days (days 1, 2 and 3 of the stress period), and the controls were treated with the equivalent volume of saline solution.

2.2.2.1 Rumen contents analysis

Rumen liquid samples were collected by nasogastric intubation on days 0, 11 and 22 of the Dexamethasone treatment period. The steers were restrained in a crush, then a flexible stomach tube (Equivet Stomach Tube, JørgenKruuse A/S, Langeskov, Denmark) was inserted through the nostril down to the rumen and aspiration of ruminal liquid was performed by creating a vacuum using a 150 ml syringe until rumen liquid flowed freely by syphon effect. Rumen contents were filtered through four layers of muslin, then 5 ml of the strained liquid fraction was added to 10 ml of PBS-glycerol (see Appendix 2.2 for PBS-glycerol composition). Samples remained in an icebox until the last animal in the pen was sampled, then pen samples were moved to the freezer and kept frozen at -40°C until further analysis. This procedure has been successfully used in many studies at this research centre (e.g. Rooke et al., 2014).

The DNA extractions were performed employing a protocol adapted from Yu and Morrison (2004) using DNA Blood Midi Kits (QIAGEN, Dusseldorf, Germany) for automated DNA extraction using QIASymphony SP (QIAGEN, Dusseldorf, Germany). DNA was quantified using a nanodrop spectrophotometer (Thermo Scientific, Massachusetts, USA) and quality checked by automatic electrophoresis on genomic screen tape using Tapestation (Agilent, California, USA). Extracted DNA was processed at The Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign for Fluidigm sample library preparation and Illumina sequencing. Primers targeting the V3–V5 region (F357 and R926) were used to amplify a region of 570 base pairs of the bacterial 16S rRNA gene. Archaea-specific primers Arch349F and Arch806R were used to amplify a 457-base-pair 16S rRNA gene fragment. Fungi specific primers for the internal transcribed spacer region (ITS3/ITS4) in the rRNA operon were used as well. For eukaryotes, the 18S rRNA gene was used as a target for the specific primers (F566Euk/R1200Euk). The amplicons were sequenced on one HiSeq flow cell.

2.2.2.2 Faecal cortisol

Faecal grab samples were obtained when animals were weighed and restrained for other purposes every two weeks during the pre-treatment period and on days 1, 2, 3, 4, 11, 15 and 22 of the treatment phase. These were refrigerated immediately after collection and kept frozen until analysis. Cortisol metabolite analysis for 11,17-dioxoandrostanes was performed on these faecal samples as per the methods described by Palme and Mostl (1997) and Palme et al. (1999). Briefly, samples were first homogenized, then 0.5 g of faeces were placed in 5ml of 80% methanol. After shaking these samples for 30 minutes in a multi-vortex, they were centrifuged for 15 minutes at 2500 *g*. The supernatant was collected and preserved by freezing at -40°C. The concentration of cortisol metabolites (11,17-dioxoandrostanes) in these samples

was determined using a group-specific enzyme immunoassay (11-oxoaetiocholanolone ELISA).

Samples from the same animal were run in the same plate to reduce plate variability for intra-animal comparisons between time points. Each ELISA plate had its own set of cortisol metabolite standards. Plate standard curves with an R^2 of less than 0.9 deemed the plate unreliable, requiring repetition of the analysis of the corresponding samples. All samples were run in duplicate. If the coefficient of variation (CV) between sample duplicates was larger than 30%, the result was deemed as invalid, and the sample was re-analyzed. The same positive control pooled sample was run in quadruplicate in all the plates to assess intra-plate repeatability of this pooled sample, as well as inter-plate consistency of the cortisol concentration of the pooled sample across different plates. Intra- and inter-assay CVs were 7.46% and 13.35%, respectively.

2.2.2.3 Behavioural activity

Activity was assessed using IceTag® sensors (IceRobotics Ltd, Edinburgh, UK), which are a tri-axial accelerometer device that can be placed on the lower leg of cattle to assess locomotion. This acceleration information is interpreted by an algorithm to express the time the animal was either lying or standing, a count of the number of steps, and a Motion Index, which is an IceRobotics proprietary index that expresses the overall activity of the steer in a given timeframe, calculated using the average magnitude of acceleration on each of the 3 axes (Kokin et al., 2014). IceTags were placed on the hind leg of the steers at the level of the metatarsus. All animals wore the Icetags for 2 weeks during the baseline period and were later placed on the control and DEX animals for the entire duration of the treatment stage (days 0-22). In addition to lying, standing, step count and Motion Index, lying bouts and standing bouts were calculated using an algorithm adapted from Tolkamp et al. (2010) to calculate bouts

based on the lying and standing episodes. This algorithm also filters out any lying bouts shorter than four minutes, since it has been established previously using video data and log-survivorship plots that this threshold is good at removing artifactual lying bouts (Tolkamp et al. 2010). The activity data was cleaned for further analysis keeping only the days the animals were undisturbed. The first two days of activity after the Icetag was placed on the animal were also removed, since other authors have suggested that animals take this amount of time to fully habituate to wearing the IceTag (MacKay et al., 2012). IceTags are sealed devices and use a non-rechargeable battery. On occasions, data was lost due to a low battery IceTag malfunction. Therefore, animals with periods of missing locomotion data had to be removed from the activity analysis. For this reason, the activity analysis was based on a smaller sample size (n=35).

2.2.3 Statistical analysis

Statistical analyses were carried out using R (v3.5.2) and Genstat 16 (VSN International Ltd., Oxford, UK), while metagenomic data were analysed using QIIME2 (Bolyen et al., 2019) v2019.1.0.

The data available for feeding behaviour, locomotor activity and performance were examined for their approximation to the normal distribution using the Anderson-Darling test and transformed where necessary. Correlation tests were used to reduce the number of variables within the same category (e.g. locomotion). For those variables showing correlations above 0.8, only one was kept for further analysis and preference was given to those that did not need transformation and were less correlated with other variables.

Raw sequence reads generated by HiSeq were demultiplexed and non-biological nucleotides removed (i.e. primers and adapters), forward reads were truncated at 233

nucleotides (nts) and reverse reads at 229 nts based on quality plots generated by QIIME2. Rarefaction is commonly used to simulate an even number of sequence reads per sample. Substantial differences in sampling depth between samples could make samples appear more dissimilar than they actually are. This can affect some diversity indexes more than others depending on their weighting of rare species. Nonetheless, it is argued that rarefaction can lead to the dismissal of parts of the available data. For this reason, if rarefaction is used, it should be minimized, or alternatives sought if rarefaction may lead to discarding a lot of data (Gloor et al., 2017). In order to minimise relevant data loss, the rarefaction sampling depth was set at 81025, which was the value of the sequence count in the lowest non-outlier sample. This led to the removal of two outlier samples with sequence counts that were more than 1.5 times the interquartile range (IQR) below the first quartile.

Sequences were converted to amplicon sequence variants (ASV) abundances using DADA2 application. DADA2 is an open-source new generation amplicon clustering tool, using specific model-based approaches to infer true value composition using a divisive amplicon denoising algorithm (DADA). This allows the production of higher resolution tables of ASV with fewer spurious sequences, which are more specific and sensitive than the more traditional use of Operational Taxonomic Units (OTU) methods (Callahan et al., 2016). The OTU methods simply group sequences using an arbitrary similarity threshold (usually 97% similarity) which may lead to loss of relevant biological variation. For taxonomic analysis, we used a pre-trained Naive Bayesian Classifier (gg-13-8-99-515-806-nb-classifier.qza version 2018.6) available from the QIIME2 platform. The ASV tables were used to determine metrics for alpha (Shannon Diversity, Faith's Phylogenetic Diversity Evenness and Observed OTU) and beta diversities (weighted and unweighted UniFrac distances). Principal coordinate analysis (PCoA) was performed on alpha and beta diversity metrics using QIIME2,

then visualized using EMPeror (Vázquez-Baeza et al., 2013) to identify variables that stratified the samples, such as treatment, pen and sampling timepoint.

Analysis of Composition of Microbiomes (ANCOM) was used to assess differential abundance at species level between samples collected pre- and post-dexamethasone treatment. ANCOM is a tool to identify differentially abundant features across ecosystems (Mandal et al., 2015), or as in this case, sampling timepoints. ANCOM compares each taxon relative to the abundance of all other taxa one at a time, assessing the statistical significance on Aitchison's log-ratio transformed data (Gloor and Reid, 2016) which helps to identify those taxons that are significantly different between sampling timepoints. This tool has the advantage that it takes into account the multivariate compositional nature of the data and includes procedures to control for false detection rates (Weiss et al., 2017). To perform the ANCOM, we filtered the taxonomic abundance data for the samples pre- and post-treatment. Then the species that were not informative were removed using the Qiime2 q2-composition plugin. This led to the removal of features rarely observed (present only in 4 samples), those with fewer than 10 reads across all samples, and those with a combined abundance of less than 100 U across all samples. Then the function *qiime composition ancom* was used for the analysis.

Linear mixed models (LMM) in GenStat 16 were used to assess the contribution of the basal diet (concentrate vs forage), feed efficiency groups (high vs low RFI),treatment (Dexamethasone vs control), and interaction of these factors as fixed effects on DMI, faecal cortisol and activity parameters (Motion Index, total daily lying duration, average lying bout duration, average standing bout duration and average daily steps) as outcome variables. Pen (4 levels) and interaction of animal within pen were included as random effects. LMM were also used to assess the contribution of

sampling timepoint and the previously described fixed effects on Shannon index and within animal UniFrac distances. Statistical significance was assumed at $p \leq 0.05$ and statistical tendencies at $p \leq 0.1$ for all analyses.

2.3 Results

2.3.1 Changes in activity following dexamethasone administration

The results obtained fitting the LMM on activity parameters revealed no significant differences between the DEX treatment and control groups in total daily lying duration, average lying bout duration, average standing bout duration or average daily steps. The only activity parameter showing differences due to treatment was the average of Motion Index of the first 7 days following the start of the dexamethasone treatment ($F_{1,30}$=9.21, p= 0.005) with animals in the DEX group having lower daily values for this parameter than control animals (3327 vs 4067 SED 243.8, see Figure 2b).

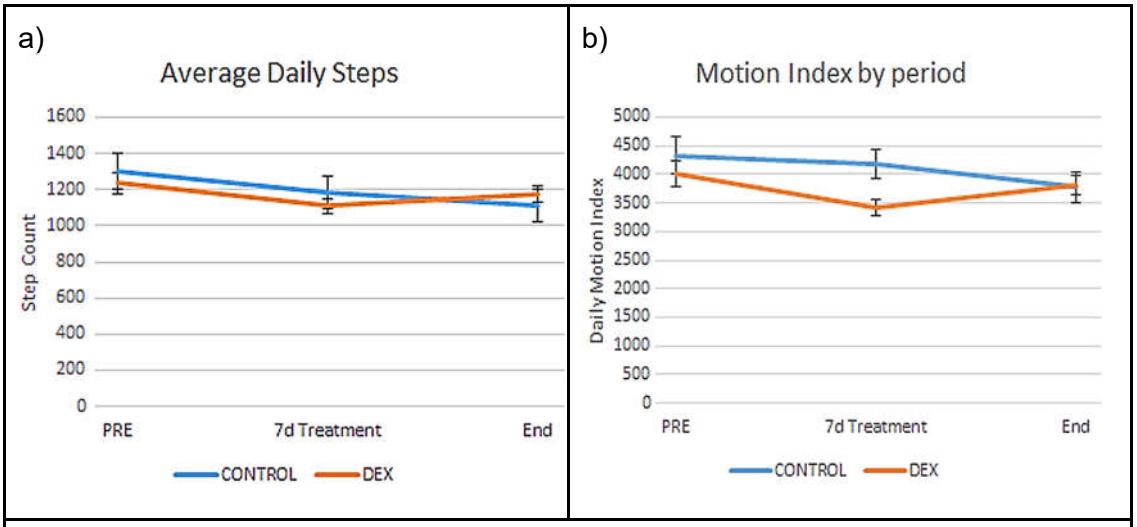


Figure 2.2. Average daily activity in the pre-treatment period, 7 days following the start of dexamethasone treatment and last two weeks of the trial (end). The graph shows no difference in the average number of daily steps (a), but Motion Index graph (b) shows a significant difference in activity acceleration for days immediately following the dexamethasone treatment. Values represent mean ± SEM

DMI did not show any significant differences attributable to dexamethasone administration. Nonetheless, other explanatory variables did have a significant effect on DMI, as was the case for diet ($F_{1,35}$=9.66, p= 0.004), where animals allocated to the concentrate diet showed a higher intake (12.22 vs 10.98 SED 0.613 kg/day), as

well as feed efficiency ($F_{1,35}$=25.82, p=<0.001), where more efficient animals showed a lower intake (10.63 vs 12.57 SED 0.391 kg/day).

2.3.2 Effects of dexamethasone on faecal cortisol metabolites

The results based on the LMM indicate that faecal cortisol values were affected by diet ($F_{1,359}$=217.77, p<0.001), sampling day ($F_{10,359}$=5.31, p<0.001) and an interaction of sampling day and treatment ($F_{11,359}$=1.94, p=0.034). Animals on the forage-based diet showed consistently higher faecal cortisol values than animals on the concentrate diet (173.4 and 43.9 SED=8.531 pg/ml respectively). As shown in Figure 2.3, control and DEX animals did not significantly differ in faecal cortisol before dexamethasone administration. However, after the administration of dexamethasone (days 1, 2 and 3), faecal cortisol was significantly reduced in the DEX group compared to the control group. This effect signals that levels of circulating glucocorticoids due to dexamethasone administration were high enough to induce central negative feedback on cortisol production. These differences in faecal cortisol became non-significant by 11 days after the start of the dexamethasone treatment.

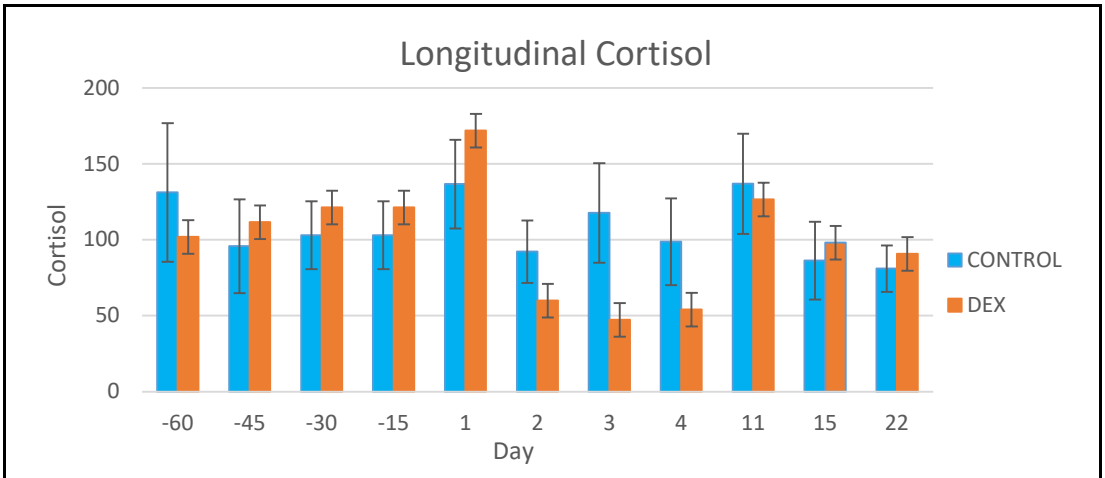


Figure 2.3. Longitudinal change in faecal cortisol. Cortisol metabolites (11,17-dioxoandrostanes) levels are shown on the y-axis as pg/ml. Days are labelled relative to the start of the dexamethasone treatment on day 1. Cortisol post dexamethasone was significantly different between DEX and Control (days 2, 3 and 4). Values represent mean ± SEM.

2.3.3 Effects of dexamethasone on the rumen microbiome

The analysis was based on 120 samples, with an average of over 680,000 reads per sample. No significant differences between the treatment groups, feed efficiency groups, or sampling timepoints were found for the archaea: bacteria ratio. However, differences attributable to diet were found ($F_{1,\ 112}$=64.52, p<0.001), which were consistent throughout the study (see Figure 2.4).

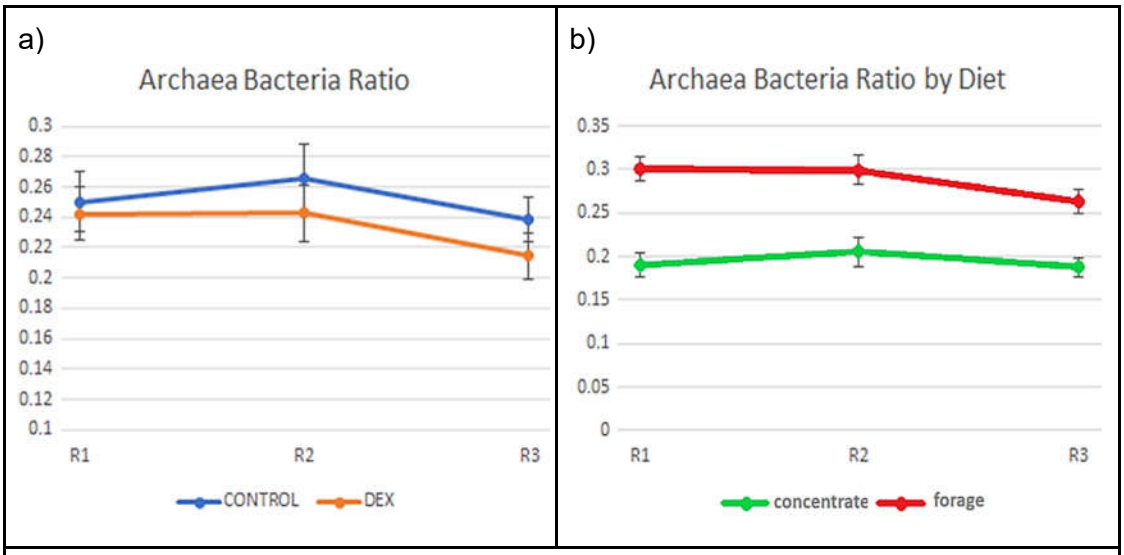


Figure 2.4. Archaea-Bacteria ratio at each of the rumen sampling timepoints. Pre-treatment rumen sample (R1), 7 days after the last dexamethasone injection (R2) and end of the trial (R3). The graph shows no difference in archaea-bacteria ratio between treatment groups (a) and consistent significant differences between the two diets (b).

At the phylum level, the composition of the rumen microbiota did not show any significant effect due to dexamethasone at each of the sampling timepoints post treatment. This finding is evidenced in Figure 2.5, where visual inspection of relative abundances of phyla at the three rumen sampling timepoints remains similar for treated and untreated animals.

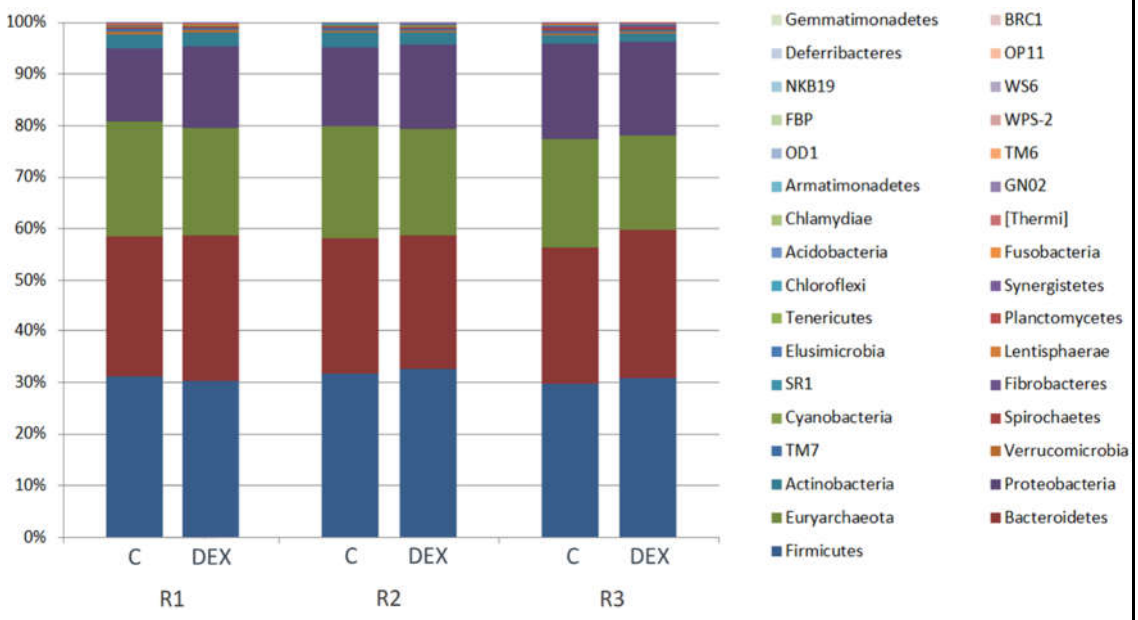


Figure 2.5. Relative abundances at phylum level for DEX and Control (C) groups at the three rumen sampling timepoints. Pre-treatment rumen sample (R1), 7 days after the last dexamethasone injection (R2) and end of the trial (R3). No significant differences were found.

2.3.3.1 Diversity Analysis

Alpha diversity is used to measure the variance in taxonomic groups within a particular sample. Therefore, it can provide metrics to assess the number of different species present and homogeneity in abundance of the different species in a sample. A common alpha diversity metric to assess the evenness of microbial communities in a sample is the Shannon Index, which in this experiment showed consistency through different sampling timepoints. The results fitting LMM to assess the Shannon index diversity as a repeated measure, found differences between treatment groups ($F_{1,\ 32.3}$=5.03, p=0.032), but this was not attributable to the treatment itself, as sampling timepoint showed no significant effect (see Figure 2.6). This indicates that the treatment groups had different means from the beginning of the study, but the dexamethasone treatment did not induce changes in alpha diversity (see Figure 2.7). Most important differences were attributable to diet ($F_{1,\ 31.8}$=186.36, p<0.001) with the

forage-based diet leading to a higher Shannon index than the concentrate diet (7.832 vs 6.594 SED 0.1434). Pen had a significant effect ($F_{2, 31.8}$=3.58, p=0.04), but it was also confounded with diet as each diet had two replicates. No difference was found in diversity between the RFI groups. However, a statistical tendency was identified for interaction between sampling timepoint and RFI group ($F_{2, 71}$=2.78, p=0.069) with animals of low feed efficiency having significantly lower diversity at timepoint 2 than more efficient animals at timepoint 3 (difference 0.41 LSD 0.30). This was independent of dexamethasone treatment, and the effect size was relatively small.

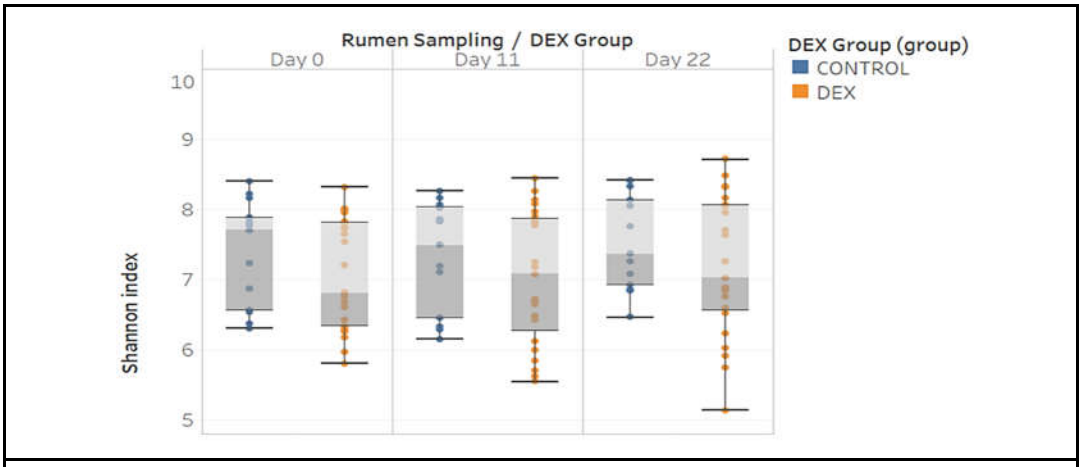


Figure 2.6. Distribution of Shannon Index for DEX (orange) and control (blue) groups for each of the sampling days. Each dot represents an animal in that group. Distribution is relatively stable and similar between groups.

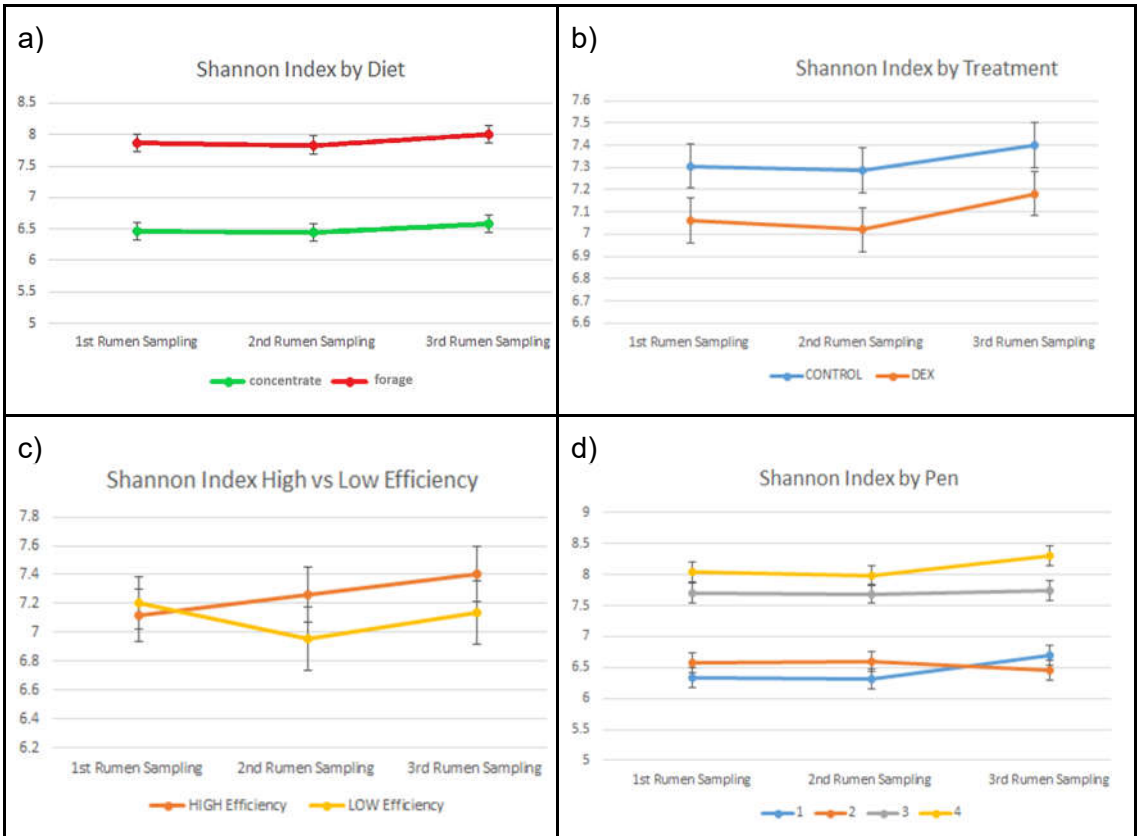


Figure 2.7. Shannon Index of diversity at each of the rumen sampling timepoints. Shannon diversity was plotted against (a) Diet, (b) Dexamethasone treatment, (c) Efficiency category and (d) Pen. There were significant differences between treatment groups, diets and pens, but these were consistent over time and not an effect of the dexamethasone treatment. A statistical tendency was identified for an interaction between time and RFI group (c).

Further to this, we examined the dexamethasone effect, focusing on the samples from the DEX group at the pre- and post-treatment timepoints. These samples were analysed against four standard alpha diversity metrics by plotting the Shannon Diversity, Faith's Phylogenetic Diversity, Evenness, and Observed ASV count as axes on Principal Coordinates Analysis (PCoA) plots (See Figure 2.8). Data were coloured based on sampling timepoint using only the first two timepoints (pre- and post-treatment), and clustering as a result of dexamethasone treatment was not evident for any of these metrics (Figure 2.8), showing that for animals in the DEX group, pre-treatment and post-treatment communities did not differ in alpha diversity.

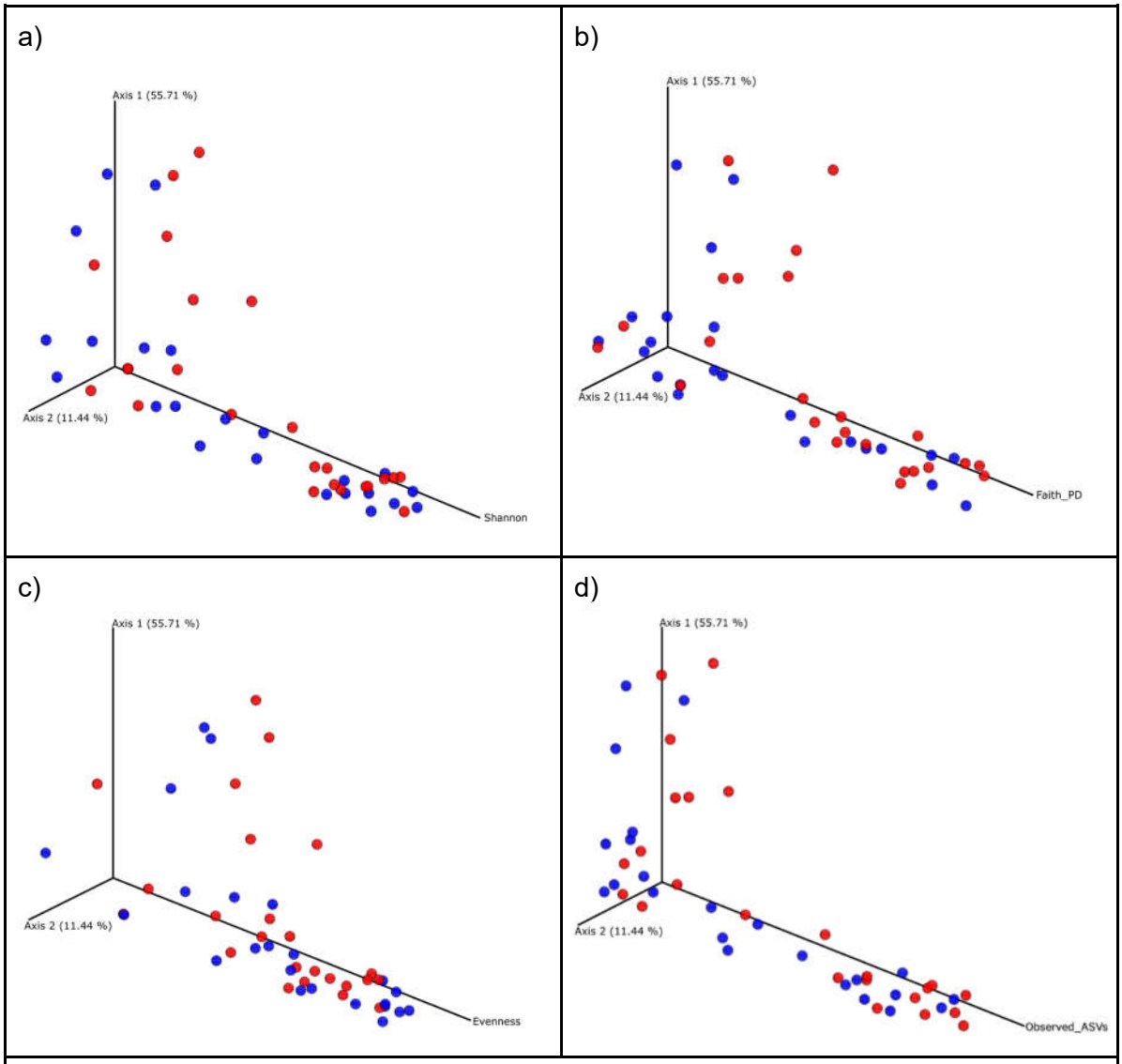


Figure 2.8. Diversity PCoA plots for DEX animals at Pre-treatment and Post-treatment. Metrics used correspond to (a) Shannon Diversity, (b) Faith's Phylogenetic Diversity, (c) Evenness, and (d) Observed ASV counts. Axis 1 and 2 are principal coordinates describing 55.71% and 11.44% of the variation in the data. Red dots represent rumen sampling before treatment, and blue dots represent post-treatment samples. There is no defined separation between the sampling timepoints that could be attributed to changes due to dexamethasone treatment.

In contrast to alpha diversity, beta diversity measures the differences in microbiota composition between different sites or samples. UniFrac distances are commonly used to provide information on how close two microbial compositions are, as it assesses the phylogenetic distances between the organisms found in the samples. unweighted UniFrac distances (Lozupone and Knight, 2005) are based only on the

sequence distances, assessing the relative length of phylogenetic branches that lead to microorganisms in only one of the samples compared to the overall genetic tree. In the case of the weighted UniFrac distances (Lozupone et al., 2007), these branch lengths are weighted by the relative abundances of each taxon, providing a useful quantitative measure of ecological distances between microbial communities.

The results of the LMM using only the weighted UniFrac distance between the pre-treatment and post-treatment samples for each animal as the outcome variable did not find any effect of dexamethasone treatment on UniFrac distances. The only parameter with a contribution to weighted UniFrac distance was diet ($F_{1,\ 1.7}$=36.54, p=0.037), with the concentrate diet leading to higher UniFrac distances than the forage-based diet (mean 0.9926 and 0.4821 respectively, SED=0.084). PCoA for weighted and unweighted UniFrac distances for post-treatment samples were plotted and analysed based on treatment group for further inspection of the data. The post-treatment sampling timepoint was selected as this would be the timepoint where differences between control and DEX should be the most evident. These plots did not show any clear separation or clustering due to the treatment group (see Figure 2.9).

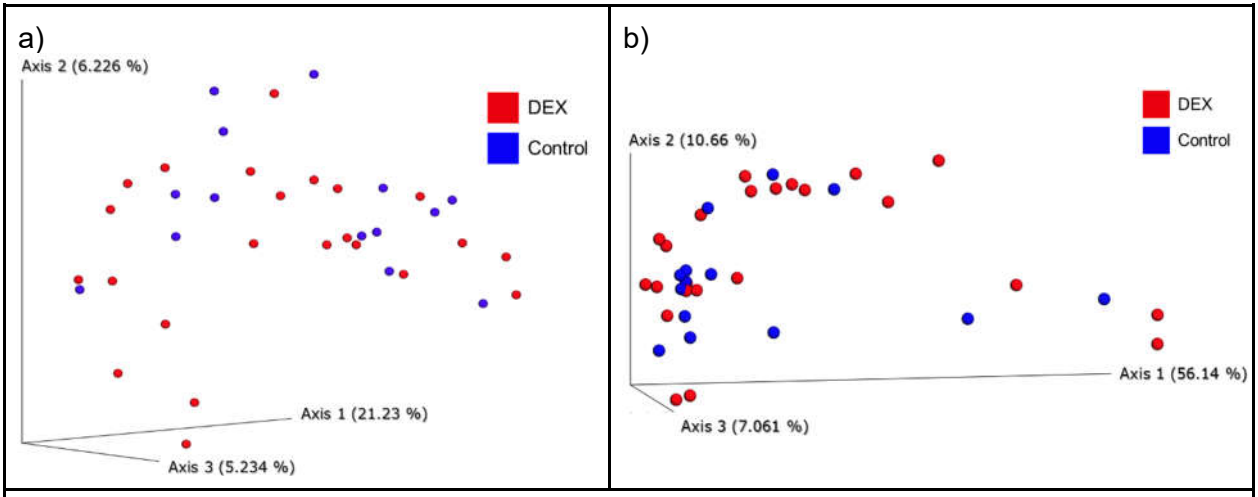


Figure 2.9. PCoA plots comparing beta diversity for pre and post-treatment timepoints. Neither figure shows separation between dexamethasone treated and control animals.

- ***a) PCoA of unweighted UniFrac distances emperor plot. Axis 1, 2 and 3 explain 21.23%, 6.23% and 5.23% of the variation.***
- ***b) PCoA of weighted UniFrac distances emperor plot. Axis 1, 2 and 3 explain 56.14%, 10.66% and 7.06% of the variation.***

Further PCoA plots were made to show the distribution of weighted UniFrac distances of the samples pre-treatment and post-treatment based on only the animals that received the dexamethasone treatment (DEX group). These plots (Figure 2.10) show no clustering by sampling timepoint, which is illustrated by the minimal variation between pre-treatment and post-treatment samples. Nonetheless, when we analysed this PCoA based on diet, there was a clear separation between groups, which can be inferred as diet having a much larger impact and explaining more of the variation in beta diversity than dexamethasone treatment (see Figure 2.10).

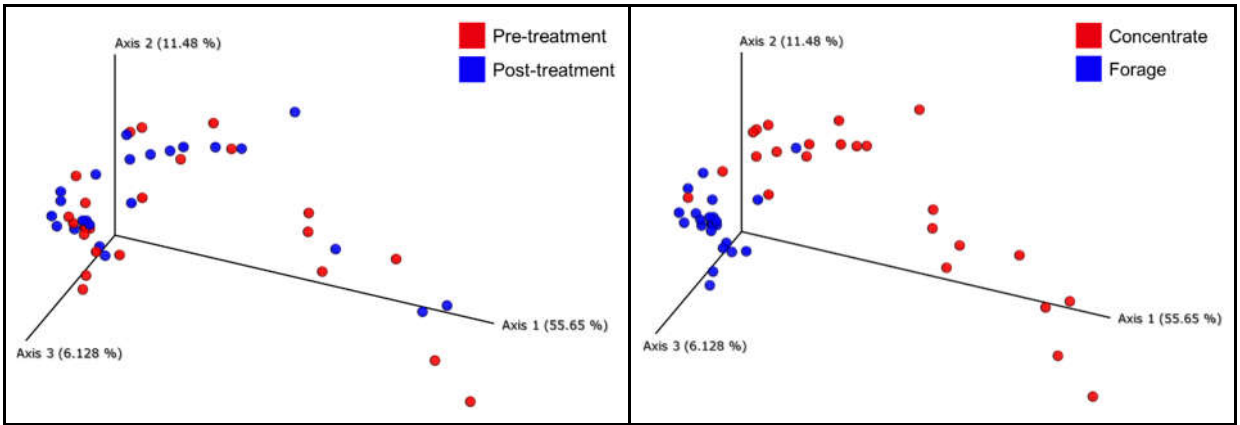


Figure 2.10. PCoA plot of the DEX animals coloured by sampling timepoint (pre-treatment and post-treatment) (left) and samples coloured by diet (right). Axis 1, 2 and 3 are principal coordinates describing 55.65%, 11.48% and 6.13% of the variation in the data. There is a clear clustering of the results based on the diet (Panel b, right), but no such clustering is observed according to pre-treatment versus post-treatment with dexamethasone (Panel a, left). It is clear from the weighted UniFrac distance that the diet of the animals (forage or concentrate) has a much greater impact on the beta diversity observed than dexamethasone treatment.

2.3.3.2 Analysis of Composition of Microbiomes

Measures of diversity can be helpful to characterize an ecosystem and identify changes, but smaller changes in one or more species can go unnoticed on a macro level. To aid in this, we evaluated changes at the species level by running an Analysis of Composition of Microbiomes (ANCOM). We used ANCOM to identify species with a difference in abundance in the rumen samples of DEX animals pre- and post-dexamethasone treatment. After removing species where the statistical power was too low to robustly estimate changes in abundance, we ran an ANCOM analysis. Figure 2.11 shows the output of a volcano plot based on the ANCOM output. In this figure, the x-axis represents the centred log-ratio transformation (clr) of the relative abundance of each species, where a more positive value in this axis would indicate that a species had a positive log-fold change compared to the average microbe. The W axis is based on the number of sub-hypotheses that have been rejected for a given species. The null hypothesis for each pairwise comparison is that the logarithmic average abundance of a given species in a sample is equal to the logarithmic average abundance of the other. Therefore, in this case, the W axis expresses the number of

times that a specific species was found to be significantly different to other species across the pre-treatment and post-treatment samples. The more times a species is different to others, the higher the chance the abundance of this species has changed between the treatments. As this is a test that relies on performing multiple pairwise comparisons looking for significant differences, no p-value is available for the W, and ANCOM itself decides what W value indicates significance (as this is dependent on the number of features tested) and returns only significant results. Only a single bacterium, *Streptococcus agalactiae,* was identified with high confidence as being differentially expressed by the DEX group in pre vs post-treatment samples (see Figure 2.11).

ANCOM Volcano plot pre-treatment and post-treatment samples of the DEX group.

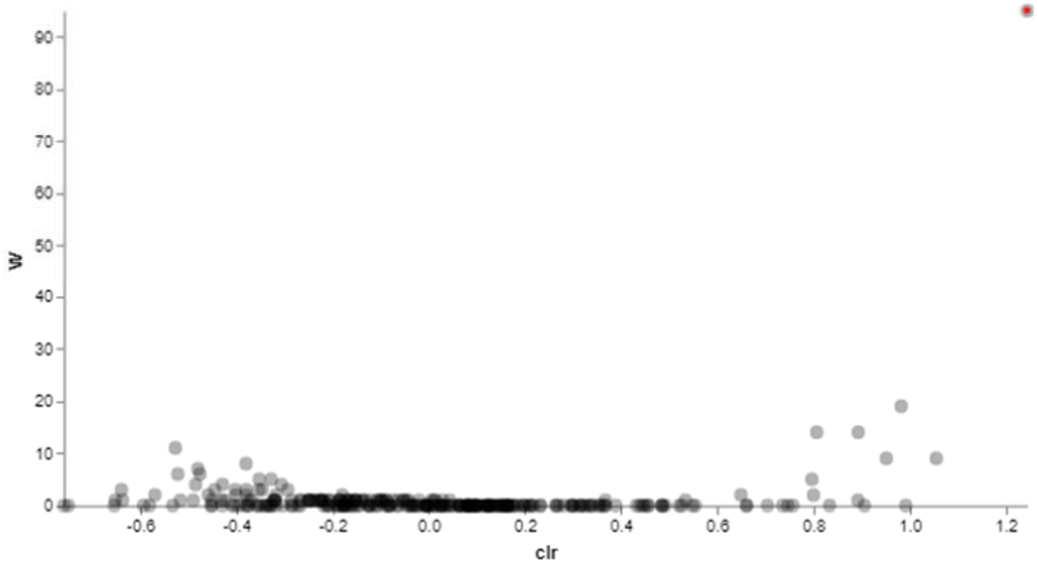


Figure 2.11. ANCOM volcano plot. The centred log-ratio transformation (clr) on the relative abundance of each species shows as the x-axis, and on the y-axis W expresses the number times the log average abundance of a species was found to be significantly different to the log average abundance of a different species between pre-treatment and post-treatment samples of the DEX group. Only one bacterium on the top right (enhanced with the colour red) is significantly different between the two sampling timepoints; this species corresponds to Streptococcus agalactiae.

Given that we cannot distinguish absolute changes in a species from relative abundance data, we therefore cannot infer directionality of the change as this is relative to the overall composition. Nonetheless, we can use the information from ANCOM as an indication of which bacteria are showing evident relative changes. To inspect this result further, we compared the relative abundance of *S. agalactiae* in relation to the control group. When we assess the relative abundance of *S. agalactiae* at the same timepoints (pre and post-treatment) in the control group, this species showed a similar change pattern (Figure 2.12), suggesting that the change in *S. agalactiae* abundance was an effect of sampling timepoint ($p<0.001$) rather than a change due to dexamethasone treatment (see Figure 2.12). Therefore, this change in abundance could not be attributed to dexamethasone administration, leading to the overall conclusion that dexamethasone administration for 3 days (0.05mg/ kg) did not lead to stable changes in the microbiome that could be detected a week after the end of the treatment.

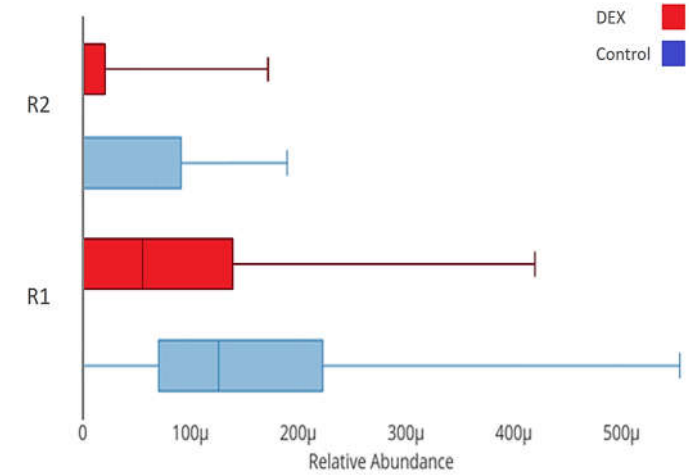


Figure 2.12. Box plot showing the distribution of Streptococcus agalactiae before and after administering dexamethasone. Sampling timepoint explains this difference in S. agalactiae abundance better than dexamethasone administration.

2.4 Discussion

The results of this study identified changes in cortisol excretion and locomotor activity due to the dexamethasone treatment, confirming that this glucocorticoid affected the physiology of these animals. However, there was no indication that the dexamethasone treatment had any significant effect on the ruminal microbial communities.

2.4.1 Effects of dexamethasone on faecal cortisol metabolites

Dexamethasone is frequently used in veterinary practice to pharmacologically mimic the effects of cortisol produced by the adrenal glands (Anderson et al., 1999), being also used to probe responses to changes in the HPA axis in research (Fisher et al., 2002; Raussi et al., 2006; Kelly et al., 2017). For this experiment, the dexamethasone dose needed to be sufficient to simulate high levels of circulating cortisol beyond the levels typically found in acute stress, while avoiding the induction of unwanted effects, particularly immunosuppression. In this experiment, the selected dose of dexamethasone (0.05mg/kg) is comparable to that used therapeutically in cattle (0.04 – 0.15 mg/kg) and significantly lower in quantity and duration in comparison to doses that could induce immunosuppression (0.2 – 0.5 mg/kg) (Papich, 2016; Ilott et al., 1997). Since dexamethasone can be recognised by cortisol target receptors (Rayalam et al., 2013), it was expected that if the dose were successful at simulating sustained high levels of cortisol, it would disturb the normal production of endogenous cortisol by triggering a persistent negative feedback on the production of cortisol.

Measurement of faecal cortisol metabolites was used to track negative feedback on endogenous cortisol release due to dexamethasone administration. The fact that the treatment group had significantly reduced faecal cortisol on the days following dexamethasone administration is an indication that the quantity of dexamethasone

administered was enough to affect this feedback system. This negative feedback occurs due to inhibition of cortisol precursor hormones at the paraventricular nucleus and anterior pituitary (Gjerstad et al., 2018). Dexamethasone has a half-life of 4.5 hours, and negligible levels are detected in plasma 54 hours after injection (Gaignage et al., 1991). In our experiment, once the exogenous dexamethasone was cleared, cortisol returned to normal showing no significant difference between treatment and control groups by day 11. Experiments by Palme et al. (1999) assessing faecal cortisol metabolites in cattle and sheep found a similar reduction pattern of faecal cortisol metabolites following dexamethasone administration.

2.4.2 Effects of dexamethasone on the rumen microbiome

Analysis of the archaea: bacteria ratio did not show significant differences between the treatment groups, only differences attributable to diet. Effects of diet in the archaea: bacteria ratio have previously been reported by others (Wallace et al. 2015b). Assessing archaea abundance and diversity is important as much research has focussed on methanogenic archaea in the rumen as it is responsible for the majority of the formation of methane (CH_4) by the reduction of CO_2 with H_2 (Popova et al., 2011). It is known that the replacement of forage structural carbohydrates with more energy-dense carbohydrates found in concentrates leads to changes in microbial profile as well as a reduction in methane emissions (Martin et al., 2010; Rooke et al. 2014). Similarly, some authors have found differences in methanogens between diets, where higher concentrate inclusion leads to a decrease in methanogens while diets with higher forage content are significantly associated with methanogenic archaea (Zhang et al., 2018; Snelling et al. 2019). These findings are consistent with the differences between diets in our experiment. On the other hand, it has been found that individual feed efficiency is not a relevant trait in determining methanogen abundance, with studies finding no differences in methanogens between

animals with different feed efficiency in cattle (Zhou et al., 2009, 2010; Jin et al., 2017) and in sheep (Shi et al., 2014). These findings are consistent with our results and highlight how the diet might be a more critical factor for methanogenic archaea abundance in comparison to residual feed intake or HPA activity.

As diet can have the most substantial effect on microbial diversity changes, the methodology for this experiment was designed to collect the post-treatment rumen sample at day 11, which is a week after we suspended the administration of dexamethasone. This number of days following administration was selected to avoid any short-term impact of dexamethasone on normal dry matter intake, which would have a sizeable effect on the microbial community composition. However, such a delay between glucocorticoid treatment and sampling creates the possibility that there were rapid transitory changes that could have reverted back to baseline before we obtained the post-treatment rumen content sample on day 11. This situation could provide an alternative explanation for the lack of differences in microbial diversity between the treatment groups attributed to the sampling methodology.

Although this is a possibility, pertinent complementary information was found from an experiment by Hua et al. (2018) in which they used a 21-day dexamethasone injection treatment (0.2mg/kg) in goats and also assessed effects on the microbiome. Their study also failed to detect changes in the microbiome even though they collected rumen content samples more frequently (days 1, 7, 14 and 21 of the dexamethasone treatment). They failed to find any effects on the microbial populations even on days during the dexamethasone administration. Although this paper is on a different ruminant species and has a smaller sample size (n=10), it still serves to inform our result, supporting our findings that there was no significant change in ruminal microbial populations attributable to the administration of dexamethasone. This

finding is in contrast to evidence found in monogastrics, where chronic stress or the addition of glucocorticoids (dexamethasone) lead to changes in the gut microbiome (Huang et al., 2015; Bharwani et al., 2016). The discrepancy between monogastrics and ruminants might stem from differential effects of stress on the gut. For example, changes in the gut microbiome of monogastrics might not be directly related to glucocorticoid circulation but rather to other compounds or other outcomes of the stress response (e.g. changes in gut motility) amongst other possible factors. If this is true, adding high concentrations of circulating glucocorticoid failed to mimic all aspects of the stress response and other components of the response may exert greater impact on the microbiome.

Another possibility as to why we did not see this clear response to glucocorticoid administration at the rumen microbial composition level might be due to the structure of rumen itself, as the rumen stems from an expanded portion of the oesophagus and is lined with keratinised stratified squamous epithelium. This epithelial tissue is not necessarily as permeable or comparable to columnar epithelium found in the lower GI tract organs in monogastric animals. Most research in monogastrics that found changes in the GI microbiome due to stress found these effects on the microbiome in locations of the lower GI tract (O'Mahony et al., 2009, 2011; Bailey et al., 2010, 2011). The lower GI tract may be more susceptible to the effects of stress in cattle. However, in living large animals, this is a difficult site to target for sampling due to the length of the GI tract.

There is also the possibility that due to the unique characteristics of the rumen as a structure specialized for anaerobic fermentation of cellulose matter, it might be more resilient to microbial community change in response to stress than the small and large intestines in monogastrics. This resilience to change of rumen microbial populations might relate to the fact that it is not in the best interests of the animal to have

substantial changes in fermentation patterns that are so relevant for survival in ruminants. Therefore, adaptive changes in the microbiome due to major changes in diet happen in a slow, steady manner. For example, Snelling et al. (2019), in a study assessing the temporal stability of the rumen microbiota in beef cattle, determined that it takes at least 25 days for the microbiome to fully adapt to a change of diet and it remains relatively stable once it has adapted. Studies that have focussed on extreme challenges to ruminal microbial communities have found that there is an internal resistance to change in these established complex microbial communities, which is attributed to microbiota developing host specificity due to selection for that specific rumen environment (Weimer et al., 2010). Even experiments performing full microbiome transplants to the defaunated rumen of a recipient cow have found that the microbial communities remain relatively similar when comparing before and 28 days after the exchange, and only a few bacterial genera might change in a small number of animals (Zhou et al., 2018), demonstrating the resilience of well-established microbiomes.

There is the possibility that other elements of chronic stress that affect behaviour, such as changes in feeding and drinking behaviour and changes in metabolism, may lead to impactful physiological changes such as altered ruminal pH. Such a change in acid-base balance, in turn, could have much larger impacts on microbial populations, but would not be directly related to high levels of glucocorticoids.

2.4.3 Other effects of dexamethasone

Activity parameters showed transitory changes due to dexamethasone administration, with dexamethasone treatment causing animals to move a similar amount but with less total acceleration (as determined by the Motion Index). This change was not evident in the number of steps alone, which might indicate a change in the type of

movement the animal performed rather than the total amount (moving in a slower fashion, or with fewer periods of high-speed movement).

There is little literature available on the effects of dexamethasone on locomotion, and a further complication is that the overall speed of locomotion is not something commonly assessed. Moreover, the Motion Index is a proprietary algorithm owned by IceRobotics; therefore, the number of studies that report Motion Index are not extensive. To the best of our knowledge, this is the first time that Motion Index has been reported to change in response to dexamethasone administration in cattle. However, it is known that common side-effects of dexamethasone may include transient lethargy and exercise intolerance (Ferguson and Hoenig, 2018), and there have been reports of reduced mean daily motor activity in mice due to dexamethasone (Katz and Carroll, 1978). Gottardo et al. (2008) describe the activity of bulls treated with dexamethasone, where these animals spent less time lying down. This finding is different from our results. Nonetheless, the results of their experiment should be viewed with care as these were based on direct scan-sampling observations over just 3 days, which may have led to inaccurate results in lying time due to the presence of people and the possibility of increased alertness due to dexamethasone treatment, rather than a direct underlying effect in daily lying times. It has been seen that in laboratory settings, a single dose of dexamethasone can induce anhedonia, which is reversible by antidepressants (Casarotto and Andreatini, 2007). Chronic dexamethasone administration may induce depression-like behaviour including anhedonia, learned helplessness, weight-loss and anxiety-like behaviour in mice (Skupio et al., 2015), and it is known that conditions characterized by chronically high levels of glucocorticoids, such as Cushing's syndrome, are commonly linked to depressive-like states that subside with correction of the underlying hypercortisolemia (McEwen, 2003). These changes in neurophysiology and motivational state could

potentially explain the short-term decrease in vigorous locomotor activity observed in our animals treated with dexamethasone.

The difference in faecal cortisol metabolites between the diets was consistent over time, which could be a systematic artefact due to moisture content in the faecal samples or due to the fibre content in the diet. In species other than cattle, some authors have attributed this effect of fibre on faecal steroid levels to the slower transit time of contents in the hindgut when animals are fed more fibre (Dantzer et al., 2011), which may lead to an increase in cortisol metabolites exchanged in the gut. For this study, the fact that the diet may have affected cortisol metabolite values did not affect our results as animals were acting as their own controls, treatments were balanced between the diets, and diet was included as a factor in the statistical models. In future studies, it would be important to consider this element, especially when comparing against previous studies or reference values. This effect also highlights the importance of considering diet as a factor when multiple diets are used, or when animals can select their diet. It might be interesting in future work to assess if the sensitivity of the 11,17-dioxoandrostanes assay used in our study is affected by either diet or moisture percentage, and how this could be corrected for comparison between studies.

Dexamethasone treatment was not found to affect dry matter intake. Comparing this information against other studies is difficult as information on the effects of dexamethasone on DMI in cattle is scarce and conflicting, as the use of dexamethasone in the differing studies varies. Dexamethasone is sometimes used in high dosages for prolonged periods to induce changes in the immune system or immunosuppression (Ilott et al., 1997; Anderson et al., 1999; Lomborg et al., 2007), which can cause inappetence. Synthetic glucocorticoids in large amounts curtail

growth rate and may lead to muscle atrophy (Courtheyn et al., 2002). However, its use in very low doses has been reported as a growth promoter for increased desirable fat deposition in the carcass (Corah et al., 1995; Vincenti et al., 2009), and having increased or no effects on DMI. In our experiment, dexamethasone was only injected over three days precisely to avoid immunosuppression or substantial changes in DMI, which could inherently affect the microbiome. An informative experiment with similarities in dosage (two IM injections of approximately 0.04mg/kg) found similar DMI and performance between treated and untreated calves (Tarantola et al., 2004). Similarly, Gottardo et al. (2008) found no difference for bulls treated with a low dose of dexamethasone (0.75 mg PO day for 49 days). Therefore, it seems dexamethasone in the doses used in our experiment should not have been expected to affect overall feed intake.

In regards to the difference in intake according to feed efficiency, lower DMI by animals having a more efficient RFI has been reported previously (Nkrumah et al., 2006; Hegarty et al., 2007; Hernandez-Sanabria et al., 2012) which is consistent with our results. There was no interaction between efficiency and dexamethasone treatment on DMI; therefore, feed efficiency is not a factor that will typically affect the intake of animals treated with dexamethasone at the dose described in our study. In regards to the effects of diet on intake, it is well established that replacing forage structural carbohydrates in the diet with non-structural carbohydrates such as those found in concentrates is associated with increases in feed intake (Martin et al., 2010), which is what was observed in this experiment.

It was found that animals with extreme differences in feed efficiency (RFI) were not differentially affected by dexamethasone administration. This was one of the aspects we were most interested in assessing, as there was the possibility that glucocorticoids could affect efficiency groups in a distinctive manner. This analysis led to the finding

that there were significant differences in diversity between low-efficiency animals at timepoint 2 and high-efficiency animals at timepoint 3. However, this was independent of dexamethasone treatment, and the effect size was relatively small. This led to the conclusion that diversity between different levels of efficiency was reasonably stable between both groups throughout the trial. Other authors have also reported no significant differences in the Shannon Index between high and low RFI animals (McCann et al., 2014). In contrast, Shabat et al. (2016) reported that more efficient cattle reduce their microbiome diversity over time, becoming more specialized for specific energy metabolic pathways. Our results are difficult to compare to these studies as our sampling timepoints only span across three weeks, and a more extended period might be necessary to assess this change over time reliably. Therefore, based solely on our results, no difference in rumen microbial diversity was found between high and low RFI groups.

As mentioned in the methods, the animals used in this study came from a previous observational experiment to validate intake monitors on two different diets. In research, re-using experimental animals is a viable strategy to reduce the total number of animals undergoing experimental procedures. Re-use forms part of the reduction element in the three Rs of humane animal experimentation (replacement, refinement, reduction). Re-use is feasible as long as the first procedure is mild, non-invasive, and the welfare of the animal does not become compromised by participating in the second experiment. This was carefully considered when ethical approval for this project was sought. The re-use of animals from a previous observational trial allowed a reduction in the total number of animals for both studies, which was the responsible thing to do from an ethical research perspective. Nonetheless, this did compromise the design and power of the present experiment, as the use of two extreme groups of RFI animals on two different diets reduces the

power and results in a loss of degrees of freedom in comparison to a factorial design with dexamethasone treatment only. To account for all these effects, we had to build more complex models (LMM) to account for diet and its interactions, as diet is probably the most critical driver of microbial communities. At the same time, alternative analysis strategies were necessary to limit the number of models at deeper taxonomic levels of the microbiome, which was achieved by using the ANCOM methodology (Mandal et al., 2015).

Our experiment used dexamethasone as a model to assess effects on the rumen microbiome in response to high levels of circulating glucocorticoids. Although repeated high levels of glucocorticoids (such as cortisol) is an expected feature of exposure to repeated stressors or chronic stress, we acknowledge that our model using dexamethasone does not have the full physiological and central effects that exposure to real-life stressors has. Therefore, this experiment does indicate that glucocorticoids have little direct effect on the rumen microbiome; however, more research is needed to evaluate the effects of repeated stressors and chronic stress on ruminal microbial populations.

2.5 Conclusions

Treatment with the exogenous glucocorticoid dexamethasone induced transient changes in behaviour and physiology, such as changes in activity and faecal cortisol, in beef cattle. Nonetheless, this glucocorticoid did not induce any significant changes in the rumen archaea population or microbial communities of the rumen in general. This provides some insight into the lack of direct effect of endogenous glucocorticoids in producing changes in ruminal microbial populations and suggests there could be a degree of resilience of the rumen microbiome to glucocorticoids. Nonetheless, further research is needed to investigate any effects of glucocorticoids on microbial

communities on other sites in the GI tract in beef cattle, as well as to study the effects of repeated stressors and chronic stress on ruminal microbial populations.

Chapter 3 - Quantification of the behavioural and physiological responses to a composite stress treatment.

3.1 Introduction

Stress is a state of threat, or perceived threat, to homeostasis and can be acute (short-term) or chronic (long-term or repeated). Although much research has studied the effects of acute stress in bovines (e.g. Herskin and Munksgaard, 2004; Chen et al., 2015), the effects of chronic stress on cattle behaviour and physiology are less clear. This chapter will cover the experimental design and results of an experiment devised to quantify the behavioural and physiological responses to a putative composite chronic stressor treatment by applying a series of commercially relevant stressors to growing-finishing beef cattle.

Under normal circumstances, a stressor that is recognised by the central nervous system (CNS) will trigger an acute stress response. In the first instance, this response will involve the activation of the sympathetic-adrenal-medullary (SAM) axis, which culminates in the release of catecholamines triggering a behavioural "fight or flight response". Metabolic and immune responses also occur, increasing blood pressure and heart rate and diverting blood flow and energy from vegetative functions into the musculature. The second arm of this stress response involves the CNS activating the hypothalamic-pituitary-adrenal (HPA) axis, where endocrine signals sent from the hypothalamus result in the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH causes the release of glucocorticoids from the adrenal cortex. Cortisol is the main glucocorticoid in cattle, and it has a critical function in regulating energy homeostasis during stress responses, thereby preparing the body for exertion; as well

as mediating and modulating the overall stress response (Sapolsky et al., 2000). Glucocorticoids also exert a negative feedback on the hypothalamus, hence being able to reduce the magnitude and duration of the endocrine response to stress (Mason et al., 2002).

Transient stressors are common in commercial beef farming such as mixing unfamiliar cattle, transport of animals, handling and temporary isolation from the herd. As herd prey animals, bovines are highly motivated to form and maintain groups with an established social structure. Therefore, social isolation or the disruption of the normal herd structure by mixing with unfamiliar cattle can create considerable stress for individual animals (Herskin et al., 2007; Chen et al., 2015). Much research has studied the effects of transport as an acute stressor on the physiology of cattle, given that beef cattle are likely to be transported more than once in their lifetime (Schwartzkopf-Genswein et al., 2016). It is generally agreed that under normal circumstances, if stressors are mild, sporadic and do not exceed allostatic coping mechanisms, their physiological costs are small. However, if stress exceeds what the animal can cope with, there can be significant consequences resulting in costly behavioural and physical responses in an attempt to deal with the stressors (Chen et al., 2015).

It is generally accepted that chronic stress and poor management can lead to deleterious metabolic changes that can affect the welfare, health and productivity of beef cattle (Moberg, 2000; von Borell et al., 2007; Freestone and Lyte, 2010; Burdick et al., 2011a). Although indicators of acute stress are generally well described, given the complexity and highly individualised presentations of chronic stress, finding tests or biomarkers to assess chronic stress reliably have proven more elusive (Russell et al., 2012). Some authors have suggested that in order to understand chronic stress, it is necessary not to view it as a continuous state but instead consider it as a succession of repeated acute stressors (Ladewig, 2000). For example, even when

cattle are housed in a reduced space which could be considered as a continuous chronic stressor, the environment would not exert a continuous activation of stress responses, but rather create multiple bouts of acute stressors due to increased competition for resources, lying space, discomfort and other related stressors. This model of thinking about long-term stress as a succession of repeated acute stressors has been termed chronic intermittent stress and has been suggested as a justifiable model to investigate chronic stress (Ladewig, 2000). This concept is commonly used in the form of chronic mild stress (CMS) treatments using multiple stressors to induce depression-like behaviours in laboratory animals (Remus et al., 2015; Willner, 2017) and has been used previously in production animals (Lomborg et al., 2008; Destrez et al., 2017; Holinger et al., 2018). It has been suggested that these types of chronic mild stressor treatments have analogies to the commonplace management of many farm animals, which may involve the unintentional application of stressors and alterations to the animals' normal environment at irregular intervals (Rutherford et al., 2006).

The following experiment applied a composite stressor treatment, comprised of four commercially relevant stressors for cattle, to assess its effects on behaviour and physiological responses of growing-finishing steers (described in this chapter), as well as evidencing any changes in the rumen microbiome, feed efficiency and methane emissions of these animals (described in Chapter 4). Given how ubiquitous the chosen stressors are in beef cattle production, the commercially relevant stressors used were reduced space allowance, in addition to being subjected every week to regrouping, transport and a short period of isolation.

In order to evidence any stress responses, we evaluated the effects of this challenging environment on plasma and faecal cortisol concentrations. Additionally, we performed

an ACTH challenge test given that prolonged or repeated exposure to stressors can alter the sensitivity of the adrenal glands to ACTH (Mormède et al., 2007). The ACTH challenge test evaluates the responsiveness of the adrenal glands, by measuring the cortisol produced in response to a known dose of synthetic ACTH. This test is commonly used to evaluate stress responsiveness in cattle in welfare research (Mormède et al., 2007; Trevisi and Bertoni, 2009).

The behavioural responses to the composite stressor treatment were assessed through several routes (summarised in Table 3.1). Temperament in cattle is generally used to describe the consistent response of an individual animal to human handling or novel environments (Fordyce et al., 1988). Temperament has been found to affect some productive parameters in beef cattle such as weight gain (Hoppe et al., 2010), dry matter intake, (Cafe et al., 2011) and meat quality (Kadel et al., 2006). It has been suggested that temperament might be related to productivity because it is an indicator of how well animals cope with stressors, and these, in turn, affect metabolism (Petherick et al., 2009). Therefore, given that individual temperament could play a role in responses to the composite stressor treatment, we assessed this as well.

Chronic stressors such as regrouping and reduced space allowance can have significant impacts on the behaviour of cattle, such as increasing agonistic interactions, reducing lying time and affecting feeding behaviour (Mounier et al., 2005; Fregonesi et al., 2007; Val-Laillet et al., 2008). Therefore, we assessed if there were any changes in behaviour in the home pen, locomotor activity and feed intake in response to the composite stressor treatment. Furthermore, we quantified the behavioural responses of steers to a novel and threatening stimulus in an attention bias test. Repeated exposure to stressors can shift the amygdala towards more sensitive unspecific processing leading to a neural hypervigilance state (van Marle et al., 2009; Henckens et al., 2016). The amygdala is a crucial regulator of emotional

processing and vigilance; it is involved in initiating the stress response and in stress sensitivity (Phelps and LeDoux, 2005). This neural hypervigilance state can lead to animals showing increased attention towards potentially threatening stimuli when they first encounter them. Hence, attention bias tests have been used to evidence negative affective states by quantifying attention to a novel threatening stimulus (Crump et al., 2018; Campbell et al., 2019). This test has been used previously in beef cattle (Lee et al., 2018) and Lee et al. (2016) validated this approach assessing attention to a threat (presence of dog) in sheep treated with an anxiogenic versus an anxiolytic drug. As predicted, animals in the anxiogenic treatment showed more vigilance and attention to the threat. In our experiment, I examined if a composite stressor treatment impacts the behaviour of beef cattle as measured through an attention bias test to a novel and threatening stimulus.

This chapter will fulfil the first objective of the experiment, which was to quantify the behavioural and physiological responses to the composite stressor treatment based on the previously described commercially relevant stressors.

Table 3.1. Summary table of key study measures and justification.

Measure	*Justification for measurement*	*Relevant references*
Cortisol (plasma and faecal)	*Cortisol is a key mediator of the stress response; has been used in the past to evaluate changes in stress responses consequence to stressors of the type applied in this study.*	*Friend et al., 1979; González et al., 2003; Huzzey et al., 2012.*
ACTH Challenge	*This test has been used in the past to evaluate responsiveness of the adrenal glands in cattle welfare research.*	*Fisher et al., 1997a, 1997b; Ladewig and Smidt, 1989; Mormède et al., 2007; Trevisi and Bertoni, 2009.*
Temperament	*Temperament can affect some productive parameters in beef cattle and serve as a trait that indicates how well animals cope with management stressors.*	*Cziszter et al., 2016; Cafe et al., 2011; Hoppe et al., 2010; Llonch et al., 2018b; Petherick et al., 2009.*
Locomotor activity	*Stressors similar to those used in this study have been found to produce behavioural changes that affect locomotor activity (such as reduced lying time and increased activity).*	*Fisher et al., 1997a.; Krawczel et al., 2012; Munksgaard and Simonsen, 1996; Telezhenko et al., 2012.*
Home pen behaviour	*Stressful environments and management stressors can induce changes in agonistic and affiliative behaviours.*	*Collings et al., 2011; Hasegawa et al., 1997; Krawczel et al., 2012; Lobeck-Luchterhand et al., 2015; Mounier et al., 2006; Napolitano et al., 2009.*
Attention bias test	Repeated exposure to stressors can induce a hypervigilance state; attention bias tests have been used to identify behavioural responses consistent with this hypervigilance when exposed to a potential threat.	*Crump et al., 2018; Ede et al., 2019; Lee et al., 2016; Lee et al., 2018; Monk et al., 2018.*
Feed intake	Some stressors have been found to affect feed intake, and altered feeding is commonly identified as an early indicator of sickness or a stressful environment.	*Collings et al., 2011; Grant and Albright, 2001; Llonch et al., 2018a; Llonch et al., 2018b; von Keyserlingk et al., 2008.*

3.2 Materials and methods

The animal trial described in the next two chapters applied a composite stressor treatment to a group of steers (castrated males). It assessed the effects of such treatment on behaviour and physiological responses (Chapter 3), as well as evidencing any changes in the rumen microbiome, feed efficiency and methane emissions (Chapter 4). The experimental design is explained in detail in this chapter, whilst the justification, methods and results of the metagenome sequencing will be covered in Chapter 4.

This experiment was approved by the Animal Experiment Committee of SRUC and was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 (PPL 70/8629). The study was carried out at Easter Howgate Farm (Midlothian, UK) from May to November 2017.

3.2.1 Animals and study design

A total of 64 homebred growing/finishing steers were used in this experiment. Half of these animals were crossbred Aberdeen Angus (AAx), and the remaining half were crossbred Limousin (LIMx) steers. The mean age at the beginning of the trial was 400 (SD 13) days. All steers were sourced from within the research farm to reduce environmental factors on the experimental animal population. All animals received the same diet *ad libitum*, formulated as a 1:1 forage to concentrate ratio on a DM basis using whole-crop barley and a premix (exact diet details can be found in Appendix 3.1), fed using the same HOKO feeders as described in Chapter 2. The steers were housed indoors on a deep sawdust bedding.

Animals were divided into eight pens of ten animals each, half of these allocated to a composite stressor treatment and half to a control regime. The composite stressor treatment (STRESS) group was composed of 24 experimental animals in 4 pens plus

an additional 16 spare Luing and Limousin animals. As described below, these spare animals were used to impose the weekly mixing stressor. By moving the 4 spare animals into a new pen each week, social instability was created for the 6 treatment animals in the group while keeping the treatment animals in their original pen. This design allowed home pen effects to be accounted for (see Figure 3.2). The spare animals were not used in the final analysis. Another 40 animals in 4 pens were used as contemporary controls. Breed, sire and weight were balanced between pens and treatment groups.

3.2.2 Experimental treatment and phases of the study

The different phases of the protocol and the specific details of the stressors applied to the STRESS treatment animals are described below (see Figure 3.3 for a schematic of the experimental timeline).

Experimental Phases

1. Adaptation period

In the adaptation phase, animals were allocated to their corresponding pen. Over the next four weeks, all steers were trained to use the HOKO feeders and were adapted to the diet described above.

2. Baseline period

Once animals had adapted to the diet and equipment, a 4-week period was used to collect baseline behaviour and feeding patterns of cattle living at a space allowance of 8.72 m^2 per animal. Data were captured over 4 weeks with minimal intervention. During this period onwards, liveweight was monitored weekly throughout the study. Individual animal feed intake and feeding behaviour were recorded using the

electronic HOKO feeders (see Chapter 2 for more details). At the end of this period, a blood sample and rumen contents sample were taken.

3. Stress period

In the Stress period, the animals in pens assigned to the control treatment remained in the same non-stressful conditions as in the Baseline period (8.72 m^2 space allowance and minimal intervention) for the next 8 weeks. Meanwhile, the following composite stressor treatment was applied to the animals assigned to the STRESS treatment:

- **Reduced space allowance**: Pens were reduced in size, leading to animals being kept at a space allowance of 4.35 m^2 per animal for the entire duration of the Stress period. The animals kept access to the same number of feeders to minimize risk of changes in feed intake due to increased competition.

- **Mixing:** Six animals in each STRESS pen remained in their home pen for the entire duration of the Stress period (STRESS Stay animals). However, four other animals (the spare animals described above, and denoted as STRESS Rotate animals hereafter) were introduced into the pen each Monday during routine weighing, whilst the STRESS Rotate animals from the previous week were removed (see Figure 3.2). As noted above, this allowed the effect of "pen" to be estimated in the statistical analysis of data on the STRESS Stay animals, whilst data from STRESS Rotate animals were not used in the final analysis.

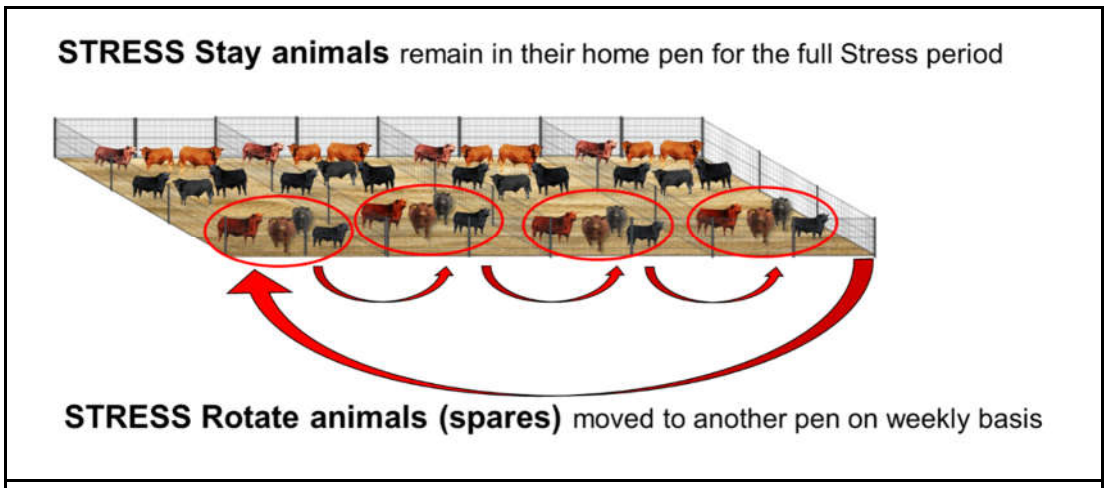


Figure 3.2. Visual description of the mixing procedure. The weekly mixing for the animals in the STRESS group was done by only moving the four STRESS Rotate (spare) animals from each pen to another pen every week. The six STRESS Stay animals in each pen remained in that pen for the entirety of the Stress period.

- **Transport:** Once per week (Tuesdays), all animals in the STRESS treatment pens were transported for 20 minutes within the farm. Animals were moved to the handling facilities and loaded into a standard cattle transporter connected to a tractor. The steers were loaded using a metal ramp. Only the animals in one pen were loaded and transported at a time. Animals were closely monitored during transport events. After unloading, cattle were returned to their home pen as quickly as possible to avoid further disturbance to feeding.

- **Isolation:** One day per week (Wednesdays), STRESS Stay animals were separated from their group mates for 10 minutes by placing them in a 4.5 m x 6 m pen with sides covered with solid panels so the animal could not see its pen mates or the outside. If an animal showed distress to the extent that it risked causing itself injury, it was released early from isolation. Therefore, any animals hitting walls, running within the pen or attempting to jump over the fence were released immediately. Every other week, a novel object was

introduced to the isolation procedure to avoid habituation to isolation. These included a yellow bucket, a blue hose, sight of a plastic bag moved by the wind, sight of an overhead bucket and a large white plastic bag traveling over the holding pen.

On the last weekday of the Stress period, a blood sample was collected as well as a rumen contents sample. On the first day of the following week, a subsample of animals was subjected to an attention bias test, which is described in detail in the study measurements section. Thereafter, on the following four days, two pens per day (one from the control group and one from the STRESS group) were subjected to an ACTH challenge, which is also described in detail in the study measurements section.

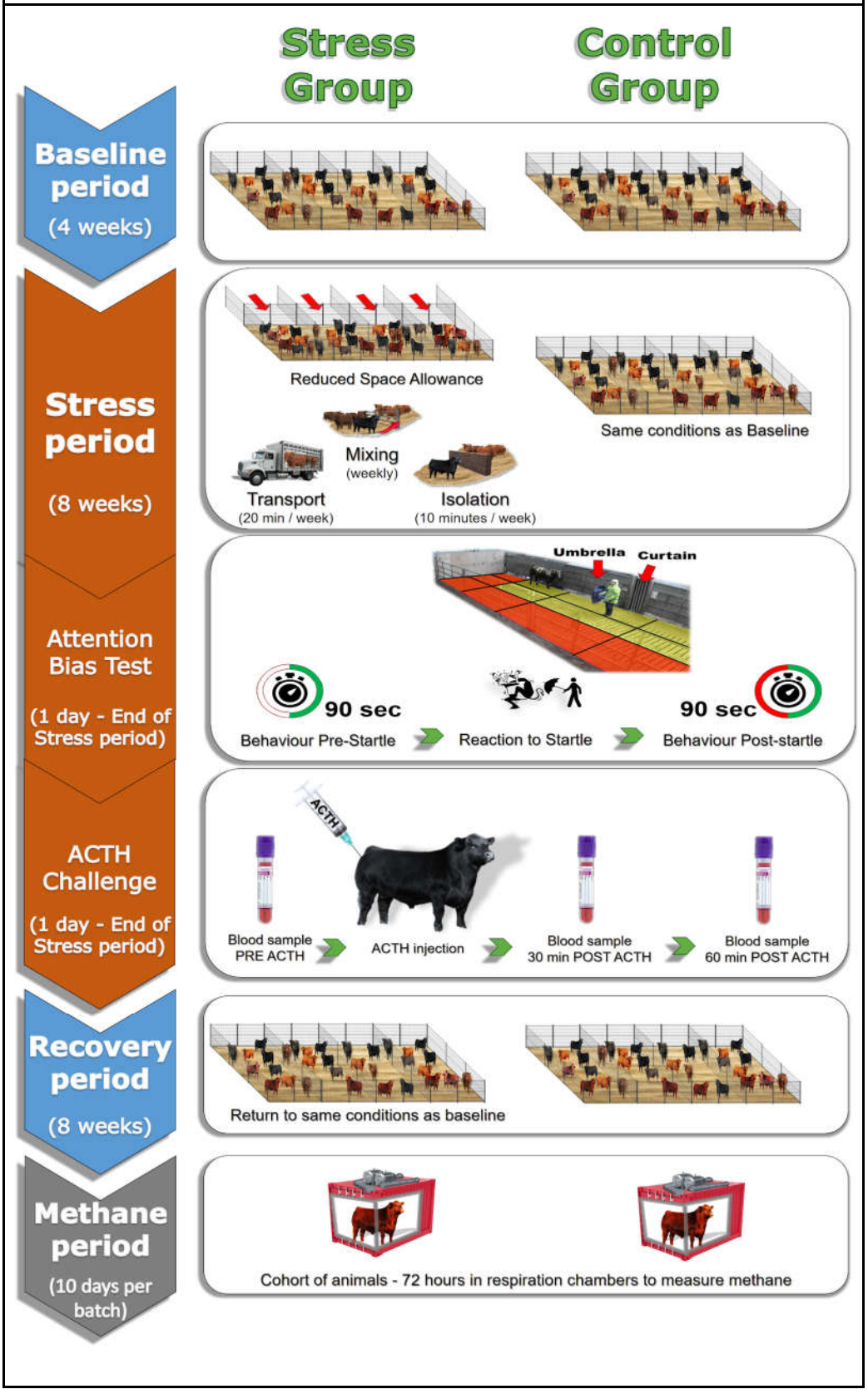
4. Recovery period

In the Recovery period, which lasted 8 weeks, animals on the STRESS treatment returned to the same conditions as they were in the Baseline period, i.e. back to a space allowance of 8.72 m^2 per animal and composite stressors (i.e. mixing, isolation, transport) were suspended. A rumen contents sample was collected on the fourth week of the Recovery period and at the end of the Recovery period.

5. Methane period

After the end of the Recovery period, methane measurements were taken from a small cohort of animals from both the STRESS group (n=6) and control group (n=6). Animals were accustomed to the chamber environment by individual housing for six days in pens with the same design as the pens used in the chambers. Following this, steers remained inside the respiration chambers for 72 hours to collect methane production parameters. More in-depth details of the methane measurement procedure will be described in Chapter 4.

Figure 3.3. Schematic diagram of the experimental design timeline



3.2.2 Study measurements

3.2.2.1 Plasma cortisol

At the end of the Baseline and Stress periods, a blood sample was obtained by coccygeal venipuncture using a vacutainer. Blood was collected in EDTA tubes and samples kept inside a cooler at 4°C until all samples from the pen were collected. Next, all samples were centrifuged at 2500 RPM for 15 minutes to separate the plasma. Plasma was then aliquoted into microcentrifuge tubes and kept frozen at -40°C until further processing.

Plasma cortisol was measured using a commercial bovine specific cortisol competitive-ELISA kit (Catalogue no. EB0062 Fine test - Wuhan Fine Biotech Co, Wuhan, China). The kit was used following the manufacturer's instructions. The plasma dilution that best covered the ranges of cortisol from our samples compared to the standard curve was a dilution factor of 1:5.

Samples belonging to the same animal were analysed together in the same plate to reduce plate variability for intra-animal comparisons between timepoints. Each ELISA plate had its own set of cortisol standards. Plate standard curves with R^2 of less than 0.9 deemed the plate unreliable, and the samples in that plate were repeated. Samples were run in duplicate; if the coefficient of variance between sample repeats was larger than 30%, the result was deemed as invalid, and the sample was re-analysed. The same positive control pooled plasma sample was run in quadruplicate in all the plates to assess the repeatability of the results and reliability across plates. For the statistical analysis of plasma cortisol, the end of the Baseline period sample was assigned as the Pre-treatment sample and the end of the Stress period plasma as the Post-treatment sample.

3.2.2.2 Faecal cortisol

Faecal grab samples were obtained every two weeks when animals were routinely weighed for the entire duration of the trial. These samples were refrigerated immediately after collection and kept frozen until analysis. Faecal cortisol metabolite analysis for 11,17-dioxoandrostanes was performed on these samples following the same ELISA methods described in Chapter 2. The faecal cortisol results available for each animal were averaged over the respective period.

3.2.2.3 ACTH Challenge

At the end of the Stress period, an ACTH challenge was imposed by performing a Synacthen test on a cohort of 41 animals. The execution of this Synacthen test was approved by Defra and the Veterinary Medicines Directorate. The Synacthen test is based on using a single injection of Tetracosactide, which is an ACTH synthetic analogue containing only the first 24 amino acids of ACTH, but with the same functional activity. This active ingredient is commonly used in veterinary medicine for ACTH stimulation tests (Fisher et al., 1997a; Papich, 2016). This approach was used to confirm any alterations in adrenal sensitivity to ACTH, which is a common consequence of chronic stress states (Wilcox et al., 2013).

For the test, a cohort of STRESS (n=22) and control (n=19) steers selected according to weight and balanced for pens and sire, were injected with Synacthen Depot (Novartis Pharma, Brussels, Belgium; concentration 1 mg/ml) intramuscularly at a dose of 0.5ug/kg liveweight (Thinh et al., 2011; Anton and Solcan, 2012). The test was performed on two pens per day (one control and one STRESS pen), taking four days to complete all pens in the trial. Blood samples to assess plasma cortisol were taken pre-treatment, 30 minutes post-injection and 60 min post-injection of Synacthen. The blood was collected by coccygeal venipuncture using a vacutainer and EDTA tubes. The same procedure previously described was used for blood

sample handling, plasma separation and storage. Plasma was analysed using the same ELISA kit and methods described in the plasma cortisol section. The day after a pen underwent the ACTH test, a rumen contents sample was collected from the steers in that pen (ACTH time point rumen sample).

3.2.2.4 Temperament Assessments

Temperament was assessed using the Crush Score (CS) and the Flight Speed (FS), described in detail below. The CS is a subjective score of the animal's response to confinement in a weigh crush. It measures the agitation during restraint and has been in the past linked to fearfulness (Grandin, 2019). Flight Speed (in m/s) is a velocity calculated from the time it takes the animal to cover a set distance as it moves away from this weigh crush once released. A faster FS has been associated with agitation during separation (Müller and von Keyserlingk, 2006). Our temperament assessment methodology differed from other studies (Grandin, 1993; Cafe et al., 2011) in that CS and FS were assessed using video footage taken when the cattle were being handled through the crush for other purposes. In order to do this, two cameras were fixed above the handling facility to video record the crush and the area beyond the crush exit.

3.2.2.5 Crush Score

The animal entered the crush, and its head was securely restrained by the yoke gate. Following this, the animal was left alone for at least 20 seconds before performing any procedures. Crush score was assessed visually from the video recordings using a modified version of the 6-point scoring system described by Turner et al. (2011a). The only modification was to score one, where the descriptor "occasional swinging of the tail" was removed from the ethogram as the tail was not observable from the camera angle. The full ethogram used for scoring can be found in Table 3.4.

All assessments were rated by only one observer. The observer was trained in using the CS ethogram by an experienced assessor. This assessor made sixty direct observations using the CS categories ethogram, and these observations were also video recorded. The newly trained observer rated these video observations, which were later compared with the scores from the experienced assessor. Scores bearing discrepancies were discussed between both observers watching the videos together. On a different day, both observers scored the video observations which were compared for interobserver reliability which was 91.6%. After this, the observer was deemed reliable to rate the rest of the videos. Each animal had an average of 8.5 separate CS records and a minimum of 6.

Table 3.4. Scoring system used by a single observer to rate the Crush Score.

Score	Descriptive Scale
1	Calm. No resistance offered.
2	Generally quiet. Offers token resistance only. Occasional and gentle movement of weight. The crush does not shake.
3	Continual movement of weight. Straining at the head restraint gate is seen. The crush does not shake.
4	Crush shakes occasionally. Animal strains at the head restraint gate and throws its head in either the horizontal or vertical planes.
5	As 4, with violent and continual shaking of the crush. The animal may fall.
6	As 5, but dangerous or unmanageable. Holding the ear to read the tag may risk handler injury. The animal may fall.

3.2.2.6 Flight Speed

Lines were painted on the fence at 3 m and 5 m from the crush exit which were used as visual guides for the video observations. The crush exit was kept clear. Other

animals were kept out of the passageway but in sight at the bottom of the handling pen.

The time taken for the animal to reach 5 m distance was recorded to the nearest tenth of a second by watching the videos at low speed. The crush exit-time was defined as the moment a leg made contact with the floor outside the crush exit, and the end time by the time taken for the animal's chest to reach the 3 m mark and 5 m mark. If an animal did not move in a straight line from the crush exit, did not complete the 5 meters or stopped halfway, the measure was deemed as invalid, and no speed was recorded. Animals had a mean of 9.3 FS records and a minimum of 3. The results for FS and CS were averaged per period to analyse change over time.

3.2.2.7 Locomotor Activity

Locomotor activity was assessed by fitting the animals with IceTag® sensors (already described in Chapter 2). The steers wore the IceTags on alternating periods of two weeks. In order to balance between the treatments, half of the animals in each pen wore the IceTags for two weeks; then the other half did so the following two weeks. This meant that activity was collected on each animal for 2 weeks during the Baseline period, four weeks during the Stress period and two weeks during the Recovery period. The information for steps, Motion Index, total daily standing duration, total daily lying duration, as well as the average duration of standing and lying bouts was filtered and calculated following the same methodology as in Chapter 2

Battery malfunction in some of the IceTags and devices falling off led to some animals having incomplete data. The final dataset used in the analysis was based on those animals with information in all three periods (n=39). For each of the activity parameters previously mentioned, the average values for Baseline, Stress and Recovery periods were calculated for use in further analysis.

3.2.2.8 Home Pen Behaviour

Two CCTV cameras were positioned above each of the home pens in order to assess any changes in agonistic and social behaviours due to the composite stressor treatment. These cameras recorded footage 24 hours a day for the duration of the study. Information on agonistic behaviours and affiliative behaviours was extracted from samples of footage. Observations were performed on only one day per week, which was selected at random from the days the animals were not disturbed for experimental procedures that week. The last two weeks of the Baseline period, eight Stress period weeks, and finally, the last two Recovery period weeks were observed for each pen, totalling twelve observations per pen. Agonistic behaviours recorded included pushing, headbutting, retaliations and withdrawals, while affiliative behaviour included social licking and social rubbing. The ethogram used to record these behaviours can be found in Table 3.5. Individual cattle were identified using unique numbers drawn on their backs using hair dye.

Although animals were fed *ad libitum*, the feeders were emptied every morning to remove rejected food and provide a new batch of fodder. The refilling of the feed bins occurred between 8:00 and 9:30 each morning. Since ten animals in each pen had only four HOKO feeders to feed from, the steers often used agonistic behaviours and assertions of dominance to displace others and access the fresh food first. This provided an opportunity to record agonistic interactions between steers. Observer XT 12 (Noldus Information Technology, Wageningen, The Netherlands) was used to record behaviours based on the ethogram. It was established from a previous experiment carried out at the same facilities that most agonistic interactions occur during the first 1.5 hours directly after feeding (Llonch et al., 2018a). Based on pilot observations, it was decided to limit the observations of agonistic behaviours to a period of 1 hour, as this included when feeders were fully and then only partially

Table 3.5: Ethogram describing behaviours observed in the home pen. Agonistic behaviours that occurred during feeding times and affiliative behaviours are grouped accordingly.

Agonistic Behaviours	**Description of Observed Behaviour**	**Recording type**
Pushing	Use of the body to physically move another steer. Contact between both bodies is made, causing the receiving steer to step away.	Frequency
Head-butting	Use of the head to physically move another steer by hitting or pushing. The head of the initiating steer makes contact with the head or body of the receiving steer and forces it to step away.	Frequency
Retaliating	The steer receiving the aggressive behaviour responds with another aggressive behaviour. This occurs immediately after the initial aggressive display.	Frequency
Withdrawing	The steer receiving the aggressive behaviour turns and moves away or steps backwards until out of range of the aggressor. This includes backing away from the feeding area, and it occurs immediately after the aggressive display.	Frequency
Affiliative Behaviours		
Social licking	The tongue of the initiator makes contact with the recipient's head or body for any length of time.	Duration
Social rubbing	The initiating steer begins rubbing the head of another steer with its own head, causing the receiving steer to rub in return, or the initiating steer begins rubbing the body of another steer with its head.	Duration

occupied. The observation time for agonistic behaviours began at the moment that the cattle were allowed access to the feeders after refilling and stopped 60 minutes later. All agonistic behaviours described in the ethogram were recorded by continuous observation of this period. In the case of affiliative behaviour, a separate two-hour

observation was performed three hours after the feeders were refilled. This timeframe was selected based on activity plots created for three pens for 48 hours. During this two-hour observation, all occurrences of social licking and social rubbing were recorded by continuous observation and the ID of both the initiator and recipient were recorded. Only one observer was used for the whole project, and actual observations began once intra-observer reliability was above eighty per cent for a sample observation of each of the behaviour categories.

The camera set up on four of the pens did not provide a good view of the feeders as these were too far away and obscured by a shadow in the morning. As the video data from these pens were not reliable, the observations for agonistic behaviour were performed in only four pens (two for each treatment). For the animals on the STRESS treatment, agonistic interactions were recorded only on the steers that did not rotate between pens at mixing (STRESS stay animals); the spare animals were regarded as "non-focal" animals. This led to a total sample size of only 32 animals for the agonistic behaviour analysis (12 STRESS group animals and 20 control animals). The affiliative behaviours did not have this issue; therefore, all eight pens could be observed, and the analysis was performed based on 24 animals for the STRESS group, and 39 for the control group.

The behavioural information available for each animal was averaged over the respective period (i.e. Baseline, Stress and Recovery periods). In the case of affiliative behaviours (licking and rubbing), due to their lower occurrence and to reduce the number of animals with a value of zero, the duration of licking and rubbing were grouped to obtain the total duration of affiliative behaviours per period.

3.2.2.9 Attention bias test

At the end of the Stress period, all the STRESS stay animals (n=24), and a balanced cohort from the control group (n=24) (matching weight and breed) underwent an attention bias test in a 4.4m x 16.3m arena (see Figure 3.6). The arena was built inside a passageway in the handling facility. Therefore, the animals had some familiarity with the place but not when socially isolated. The front and rear gate were covered with solid MDF panels to prevent the steer from seeing other animals and people outside the arena. Video cameras were placed on the rear and side of the arena. The test was composed of a pre-startle stage, a startling event and a post-startle stage, which are explained in more detail below.

A. Pre-startle stage (90 seconds)

Steers entered the arena individually through the side gate and were allowed to acclimate to the area for 90 seconds. In the middle of the arena, a black curtain covered the entrance of a small corridor; this served as a novel object stimulus in the pre-startle stage. The reactions to this "novel object" would be later extracted from video data.

B. Startling response

After the first 90 seconds, as soon as the animal faced the black curtain and stood within a 3-meter distance from it, a person wearing a high visibility jacket emerged from the other side of the curtain and opened an umbrella in the direction of the animal, startling the steer.

C. Post-startle stage (90 seconds)

After the startle response, the person remained static within the arena, and the steer remained in the arena another 90 seconds. The test was stopped immediately if an animal showed behaviours that risked injury to itself or the person in the arena

(running frantically or attempting to jump over the fence). After the 90 seconds had passed the animal was guided out of the arena and returned to its pen mates.

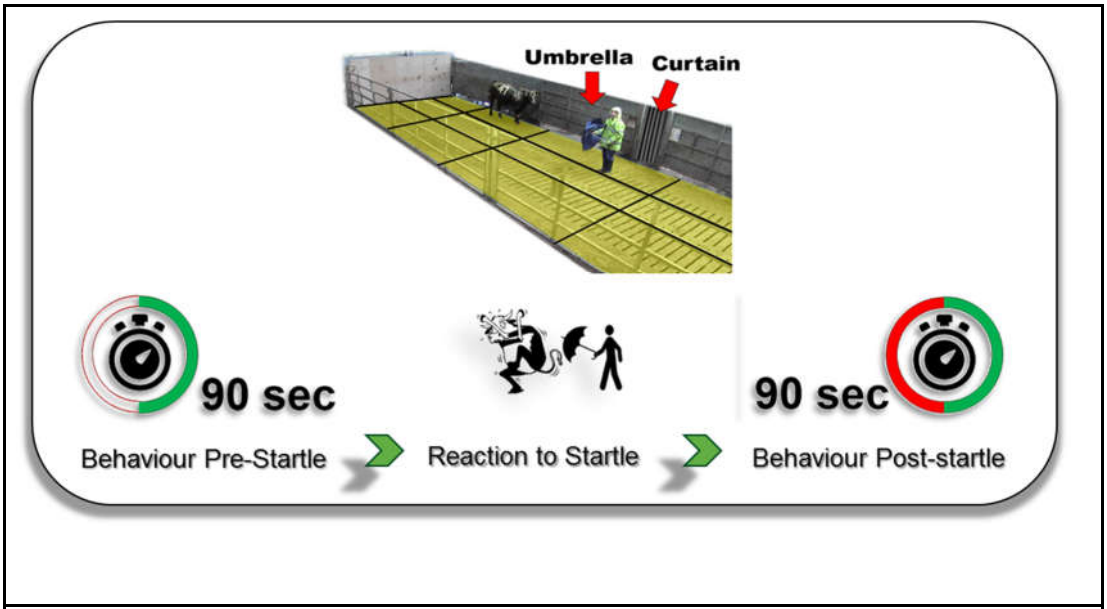


Figure 3.6. Enhanced image of the attention bias test arena and schematic of test progression. The yellow layer shows the twelve areas used to record location and area crosses.

Attention bias test video analysis

At a later date, footage from both cameras was synchronised and merged into one video to have full visibility of the animal at all times during the test. Video footage was evaluated using Observer XT 12 software (Noldus Information Technology, Wageningen, The Netherlands). The ethogram found in Table 3.7 describes the behaviour recorded from the video observations. Only one observer analysed all videos, the observations were randomised, and the person was blind to the treatments. This observer was trained on the ethogram using three observations 180 seconds long. These were observed again 3 days later, and once repeatability was over 85% per cent, it was deemed acceptable to begin observing the remaining videos.

To determine the distance of travel of the animal within the arena, a methodology by Destrez et al. (2013) to record changes in location was used. A visual grid layer was superimposed onto the video playback subdividing the arena into 12 quadrants to track movements within the arena (see Figure 3.6). This allowed the steer's location to be recorded and movement between quadrants to be used as a proxy for distance travelled.

Standing and vigilance were two different classifications, which were mutually exclusive (i.e. could not happen at the same time), describing an animal remaining immobile. Hence, a third summary behaviour adding the duration of standing and vigilance together was calculated; this behaviour was termed "immobility". The number of times a steer changed area was calculated using the location information and referred to as "Area Crosses". Some behaviour happened rarely leading to many animals having a value of zero; this was the case for touching the umbrella, sniffing, escaping, vocalisations, and tail swishing. These behaviours were not included in the analysis.

Table 3.7. Ethogram used for the behaviour recording during the attention bias test

Attention bias test ethogram		
Behaviour	**Definition**	**Type**
Walking	Animal moves slowly with three legs in contact with the floor.	Duration
Standing	Immobile. All four legs are in contact with the floor.	Duration
Running	Animal moves rapidly with at least two legs not touching the floor at any time.	Duration
Vigilance	Immobile with the head in an upright position and ears facing forward.	Duration
Sniffing	Sniffing the floor. Begins when the steer's nose touches the floor and ends when the steer's nose stops touching the floor.	Duration
Looking at novel object	Immobile; head and ears oriented towards the curtain. Only occurs during the pre-startle stage.	Duration
Looking at the threat	Immobile; head and ears oriented towards the person holding the umbrella. Only occurs during the post-startle stage.	Duration
Escape	Attempts to escape the arena by getting the head across the gate or putting the chin on the wall.	Frequency
Vocalisations	Sound made with visual confirmation of mouth open.	Frequency
Tail swishing	The tail moves rapidly from side to side three or more times.	Frequency
Touching the umbrella	The steer's nose is <10 cm from the umbrella.	Frequency
Startle response to the umbrella opening	Travel speed just after the startle of the steer: · Low startle: animal immobile after umbrella opens. · Medium startle: animal walks away. · High startle: animal runs away.	Categorical
Location	The arena is divided into 12 equal areas (1.5 m x 3.4 m). Areas are numbered from 1 to 12. The location of the steer is recorded as the area where its two front legs are located.	Frequency

3.2.2.10 Feed intake

The HOKO feeders recorded the individual visits of each animal to the feeders by detecting the read of the radio-frequency identification (RFID) of each steer; this was used to record digitally the weight of feed consumed per visit. Therefore, data from the feeders provided a daily record of the total dry matter intake (DMI) of each animal. The average DMI for each experimental period was calculated from the daily DMI. This was later used as a proxy of feed intake.

3.2.2.11 Rumen liquid samples

The procedure for collection of rumen samples was the same as that employed in Chapter 2. The specific information on sampling timepoints and information obtained from these samples will be covered in more detail in Chapter 4.

3.2.3 Statistical methods

One animal from the control group suffered a concussion unrelated to the experimental procedures halfway into the experiment and was taken off trial and its data excluded from the analysis. Statistical analyses were carried out using R (v3.5.2) and Genstat 16 (VSN International Ltd., Oxford, UK).

The data available for feeding behaviour, locomotor activity and performance were examined for their approximation to the normal distribution using the Anderson-Darling test and transformed where necessary. Correlation tests were used to reduce the number of variables within the same category (e.g. locomotion). For those variables showing correlations above 0.8, only one was kept for further analysis and preference was given to those that did not need transformation and were less correlated with other variables. Traits where satisfactory transformation could not be achieved were kept in their original unit format. Linear mixed models (LMM) in GenStat 16 were used to assess the contribution of breed, sire, treatment, period and

interactions as fixed effects on cortisol, activity, DMI and behavioural parameters as outcome variables. Pen and animal nested within pen were included as random effects. Temperament was also included as a fixed effect in analyses where other behavioural parameters were the response variables. Statistical significance was assumed at $p \leq 0.05$.

3.3 Results

3.3.1 Plasma cortisol

LMM were performed comparing pre-treatment and post-treatment cortisol as a repeated measure. These found a significant main effect of treatment ($F_{1, 57.7}$=4.28, p=0.043) on plasma cortisol, but no main effect of time period (baseline or treatment phase) and no significant interaction between treatment and time period (see Figure 3.8). Therefore, a significant difference in cortisol was present between the treatment groups even before the treatment was implemented, and this difference persisted throughout the experiment.

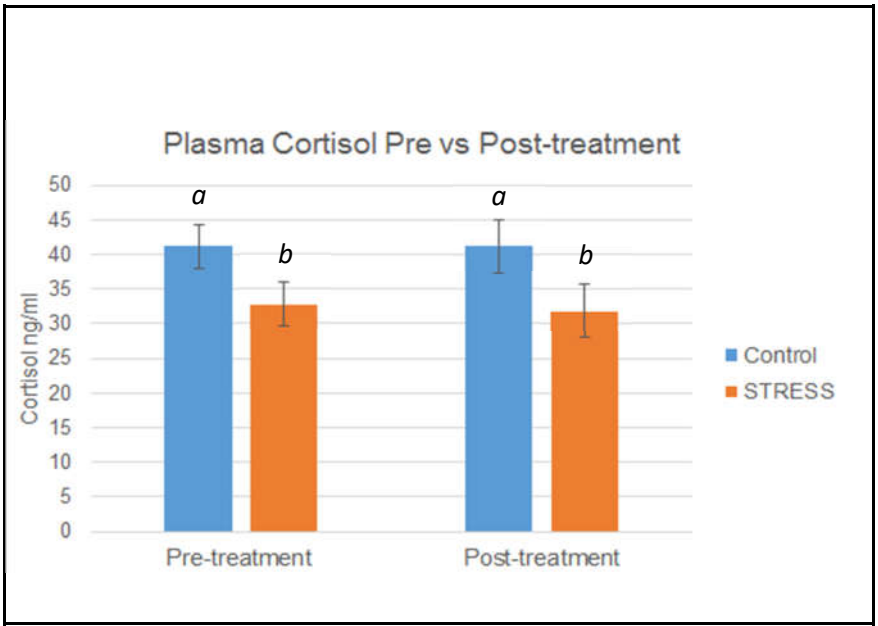


Figure 3.8. Comparison of plasma cortisol pre-treatment and post-treatment. There was consistently higher plasma cortisol for the Control group compared to the STRESS group in both pre-treatment and post-treatment timepoints, without significant changes for either group over time. Values represent mean ± SEM. Means with different letters (a,b) are significantly different (p<0.05).

3.3.2 Faecal cortisol

Faecal cortisol metabolites were higher in control animals (99.8 vs 80.56 SED 6.656 pg/ml; $F_{1, 185}$=8.43, p=0.004). There was also a main effect of period ($F_{2, 185}$=17.80, p <0.001) which meant that groups changed through time (see Figure 3.9), but there was no interaction between period and treatment. Therefore, cortisol increased over time, but these changes showed a similar pattern for both treatment groups.

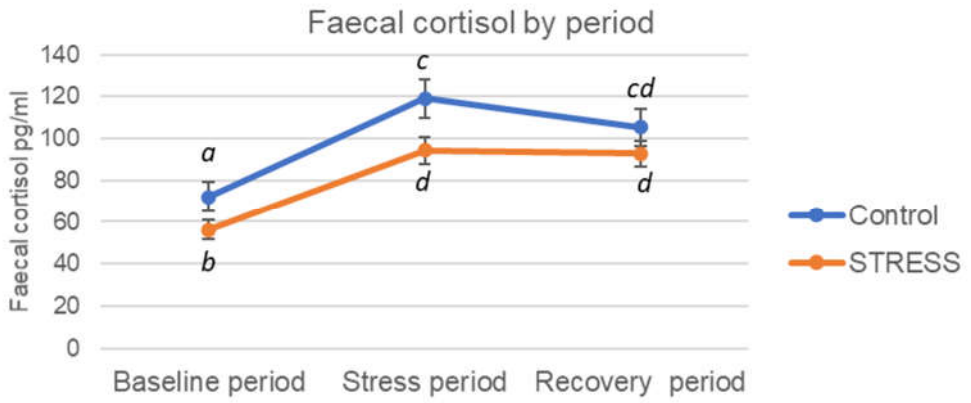


Figure 3.9. Faecal cortisol in the two treatment groups. y-axis represents concentration of cortisol metabolites (11,17-dioxoandrostanes). There were significant differences between treatment groups, but these were not specific to the period when the stressor treatment was applied (Stress period). Values represent mean ± SEM. Means with different letters (a,b,c,d) are significantly different (p<0.05).

3.3.3 ACTH challenge

There was a main effect of sampling time point (0, 30 or 60 minutes; $F_{2, 119}$=102.23, p<0.001) on plasma cortisol in response to the injection of ACTH, but no main effect of treatment and no interaction of treatment and sampling time (see Figure 3.10). The significant effect of sampling time showed a predicted increase in cortisol at 30 minutes post-Synacthen injection. Overall, these results failed to show any significant differences in adrenal sensitivity due to the composite stressor treatment.

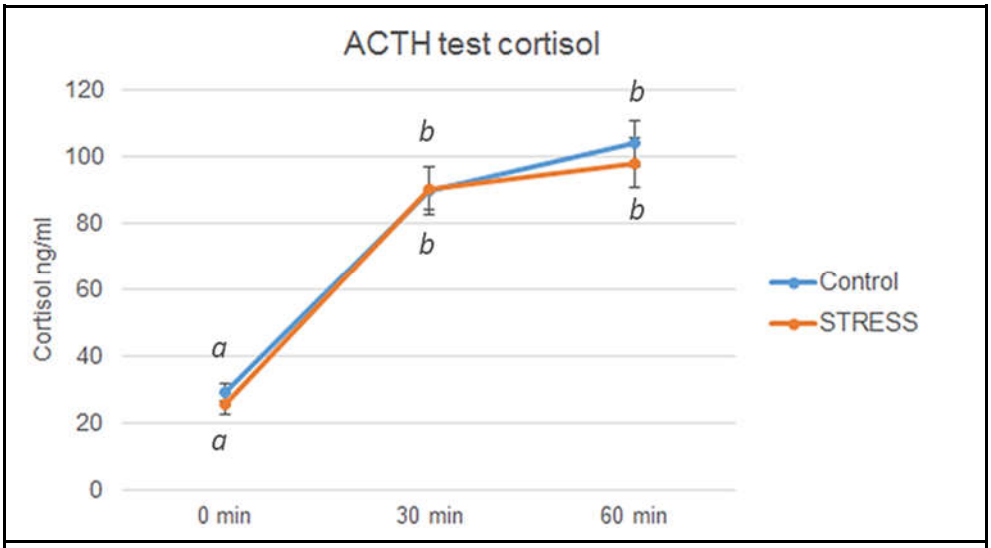


Figure 3.10. Plasma cortisol results for the ACTH Challenge. A significant increase in cortisol 30 and 60 minutes after injection occurred compared to initial values (0 min), but there was no difference between treatments. Values represent mean ± SEM. Means with different letters (a,b) are significantly different (p<0.05).

3.3.4 Temperament

There was a significant positive correlation between the crush score (CS) and flight speed (FS) temperament traits (r=0.38, p=0.05), but this was sufficiently low as to assume each trait largely measured different elements of temperament. CS was not affected by treatment, nor was there a significant variation in CS over periods. The only parameter that explained part of the variation in CS was breed ($F_{1,124}$=10.71, p<0.001), with LIMx animals having slightly higher average CS score (more agitated) than AAx (2.852 vs 2.491 SED 0.1626).

Animals in the STRESS treatment group showed faster movement in the Flight Speed test than animals in the control group (1.794 vs 1.371 SED 0.1119 m/s, $F_{1,5.7}$=13.81, p=0.011; see Figure 3.11). There was also a statistical tendency for differences between periods ($F_{1,113.1}$=3.65, p=0.059), with decreases in FS over time (1.74 vs 1.56 SED 0.095 m/s) but no interactions between treatment and period nor breed differences.

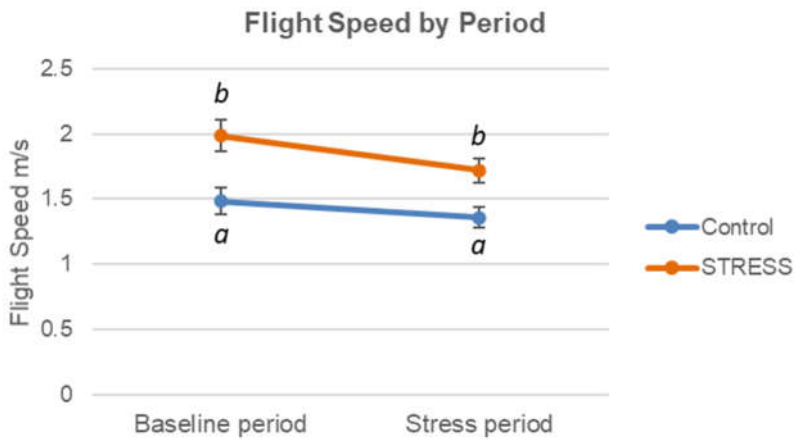


Figure 3.11. Temperament result for Flight Speed (FS). Differences in FS between treatments (significant) and according to period (statistical tendency) were found. Values represent mean ± SEM. Means with different letters (a,b) are significantly different (p<0.05).

A final overall average value was calculated for CS, as well as a final average for FS. These temperament traits were used as a covariate in the analysis of the locomotor activity, attention bias test, home pen behaviour and feeding behaviour.

3.3.5 Locomotor activity

In the case of total daily lying duration and average lying-bout duration (LBD), there were no effects of treatment, time period or their interaction (see Figure 3.12). The only effects explaining variation in daily lying duration were sire ($F_{9, 98}$=2.65, p=0.008) and FS ($F_{1, 98}$=14.96, p<0.001), with animals with higher FS having less lying time. Meanwhile, LBD was only affected by sire ($F_{9, 99}$=4.08 p<0.001).

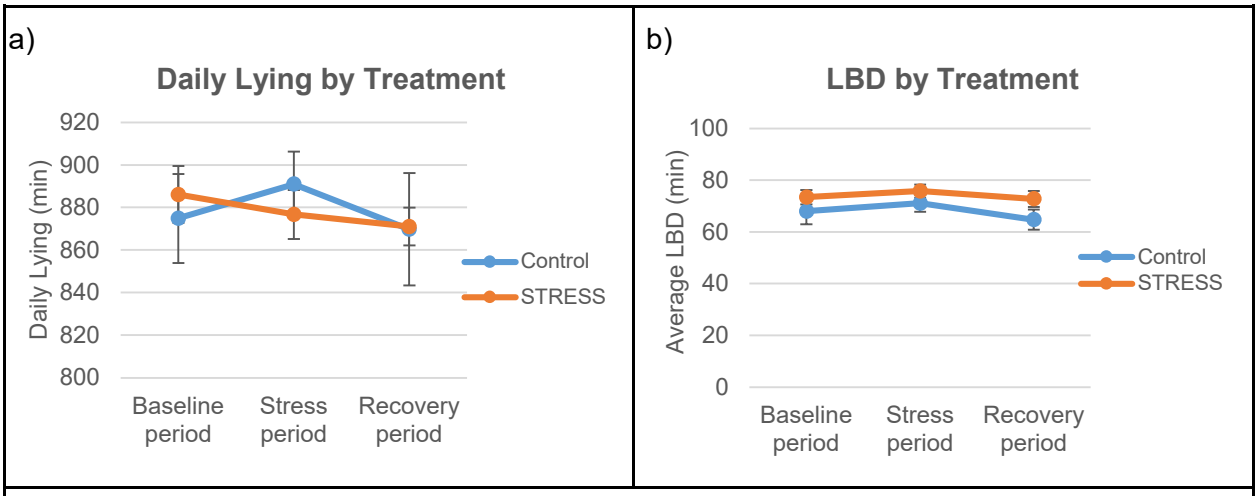


Figure 3.12. Average lying duration per period. No significant effect of treatment or period were found on (a) Average daily lying duration or (b) Average lying bout duration (LBD). Values represent mean ± SEM.

There were significant contributions of treatment ($F_{1, 95}$=12.24 p<0.001) and period ($F_{2, 95}$=13.25, p=0.001) to the number of steps (see Figure 3.13), but no interaction of treatment and period. Control animals took a greater number of steps, and both treatments showed a slight reduction in steps over time (Figure 3.13). Other factors contributing to daily steps were sire ($F_{9, 95}$=6.01 p<0.001) and CS ($F_{1, 95}$=17.19 p<0.001), which showed a positive effect on the number of Steps.

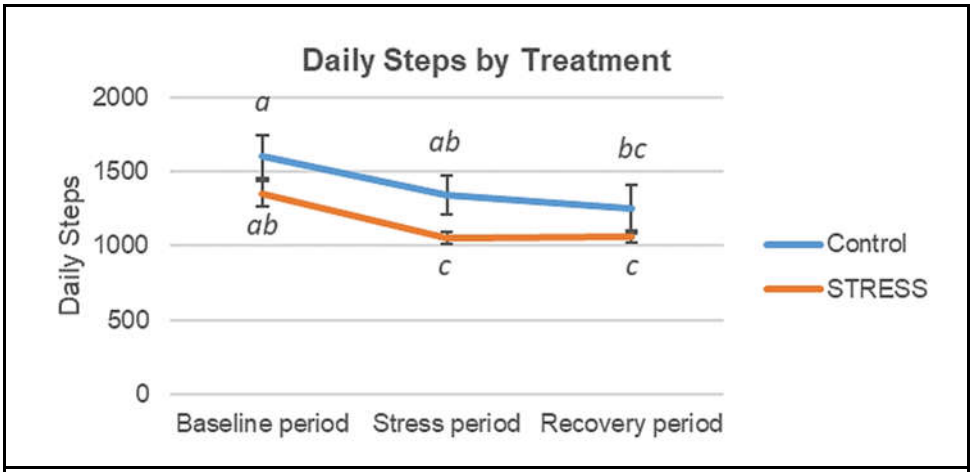


Figure 3.13. Average daily count of steps per period. There were differences between the treatments and a reduction in steps over time but no interaction. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

Average Standing Bout Duration (STBD) was affected by treatment ($F_{1, 97}$=6.80, p=0.011), but no significant differences between the periods or their interaction (see Figure 3.14). STRESS treatment animals showed significantly longer STBD than control animals (47.65 vs 43.71 SED 1.78 min). Other significant effects on STBD were sire ($F_{9, 97}$=5.36, p<0.001) and a positive effect of FS ($F_{1, 97}$=7.39, p=0.008).

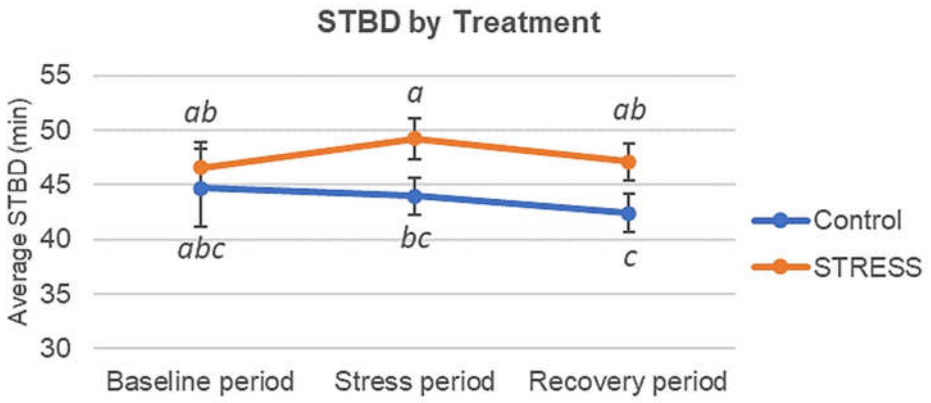


Figure 3.14. Average Standing Bout Duration (STBD) per period. There was a difference between the treatments, but no significant change over time and no interaction between treatment and period. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

Motion Index was affected by period ($F_{2,92}$=33.57, p<0.001), treatment ($F_{1,92}$=21.03, p<0.001), and the interaction between treatment and period ($F_{1, 60}$=5.48, p=0.023). Motion Index fell significantly for animals in both treatments during the Stress and Recovery periods compared to the Baseline period (see Figure 3.15). Additionally, animals in the control group showed a higher overall Motion Index compared to the STRESS group (4711 vs 3625 SED 254.6). This difference between the groups was due to significant differences between treatments specifically during the Stress period, where the STRESS group showed significantly lower Motion Index in comparison to controls (3073 vs 4845 SED 312.7). Other significant effects on Motion Index were

sire ($F_{9,92}$=6.77, p<0.001), as well as positive effects of both CS ($F_{1,92}$=15.9, p<0.001) and FS ($F_{1,92}$=6.72, p=0.011).

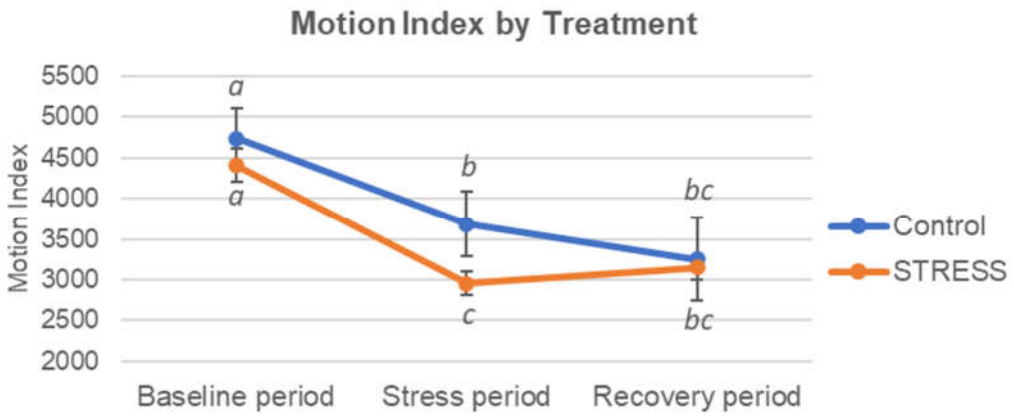


Figure 3.15. Average Motion Index per period. Motion Index changed over time and there was a significant difference between treatments during the Stress period. Y axis expresses the cumulative Motion Index. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

3.3.6 Home pen behaviour

3.3.6.1 Agonistic behaviour

The average frequency of headbutting was not affected by the treatments, but was affected by period ($F_{2,81}$=9.09, p<0.001). Animals in both treatments decreased their number of headbutts over time (see Figure 3.16). Headbutting was also affected by sire ($F_{8,81}$=2.87, p=0.007) and FS ($F_{1,81}$=5.02, p=0.028), where faster speeds were associated with less headbutting.

There were differences attributable to period on the average number of pushes ($F_{2,81}$=7.98, p<0.001), which showed a decrease over time (see Figure 3.17), but there were no significant effects of treatment nor its interaction with period. Higher FS was significantly associated with less pushing ($F_{1,81}$=6.77, p=0.011), and there was a statistical tendency for an effect of sire ($F_{8,81}$=1.92, p=0.067).

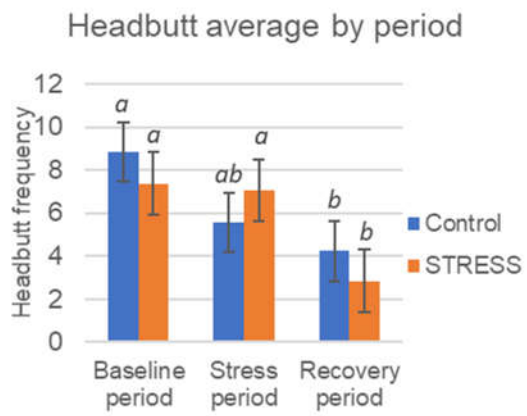


Figure 3.16. Average observed headbutts by period. There was a decrease in headbutts over time but no differences between the treatments. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

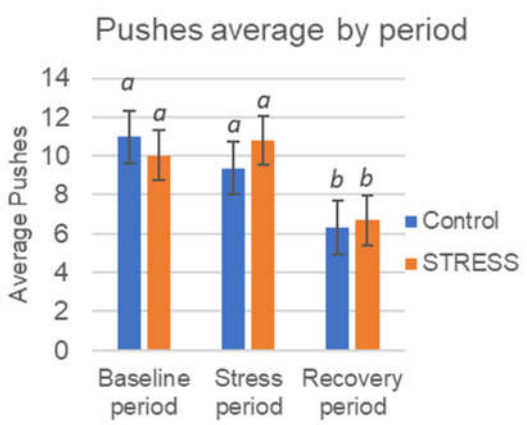


Figure 3.17. Average observed pushes by period. There were significant changes in pushing over time, but no effects of treatment. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

The number of retaliations was not affected by treatment or period (Figure 3.18); nonetheless, there was a statistical tendency for an interaction of period and treatment ($F_{5,79}$=2.11, p=0.073). When this interaction was dissected, control animals showed significantly more retaliations than STRESS animals, but only during the Baseline period (0.82 vs 0.36 LSD 0.383). Over time, the STRESS group showed an increase in retaliation during the Stress period compared to their lower Baseline value (0.88 vs 0.36 LSD 0.45). During the Recovery period, the STRESS animals returned to a level similar to their baseline. Nonetheless, the frequency of retaliations during the Stress period was similar in STRESS and control groups (0.88 vs 0.7 LSD 0.38). Sire also had a significant effect on retaliations ($F_{8,79}$=2.86, p=0.008).

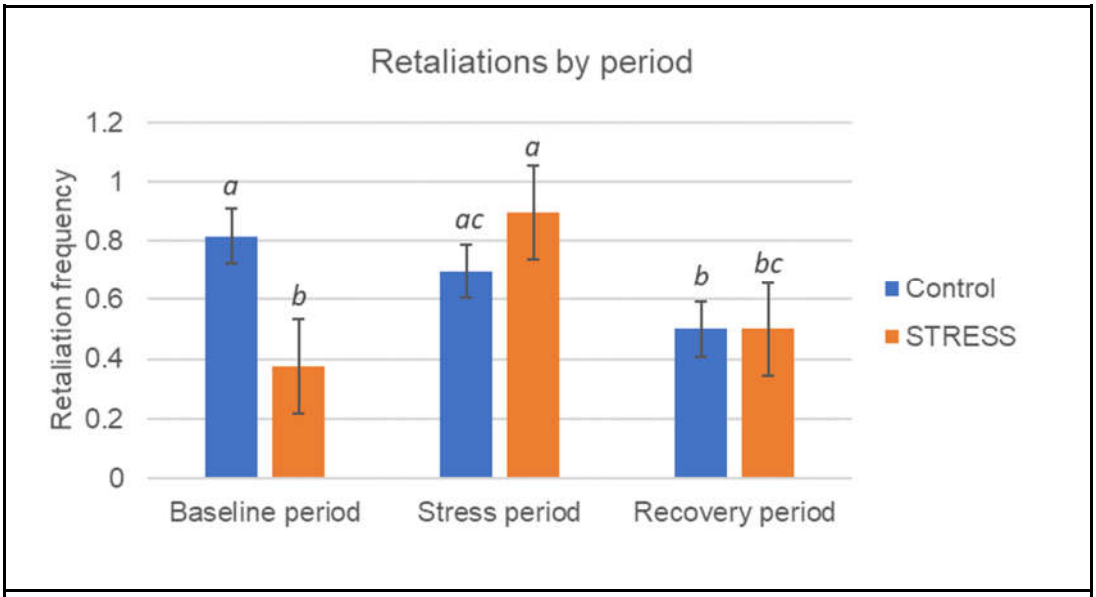


Figure 3.18. Average observed retaliations by period. There was only a statistical tendency for an interaction between treatment and period. A significant difference between treatments was present in retaliations during the Baseline which was not present during the Stress period. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

Withdrawal frequency decreased over time ($F_{2,81}$10.86, p<0.001), but there were no effects of treatment nor interactions (see Figure 3.19). Other significant effects on the number of withdrawals were sire ($F_{8,81}$=2.97, p=0.006) and a negative effect of FS on withdrawal frequency ($F_{1,81}$=18.89, p<0.001).

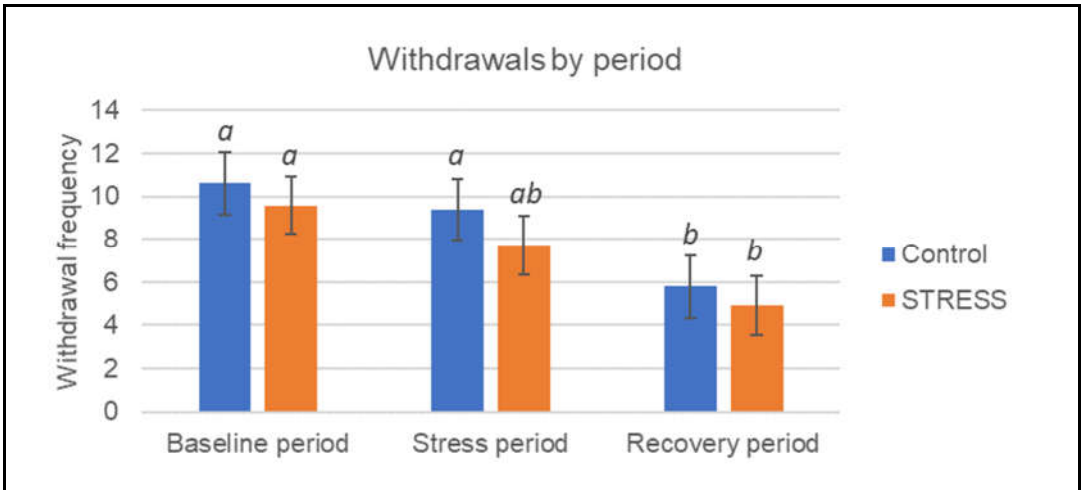


Figure 3.19. Average observed withdrawals by period. There was a decrease in withdrawals over time but no differences between the treatments. Values represent mean ± SEM. Means with different letters (a,b) are significantly different (p<0.05).

3.3.6.2 Affiliative behaviours

Due to the low occurrence of licking behaviour, duration of both rubbing and licking were grouped together as affiliative behaviours. No differences between the treatment groups, periods or their interaction were found (see Figure 3.20). Statistical tendencies occurred for sire ($F_{9,178}$=1.69, p=0.094) and a positive effect of FS ($F_{1,178}$=3.31, p=0.071).

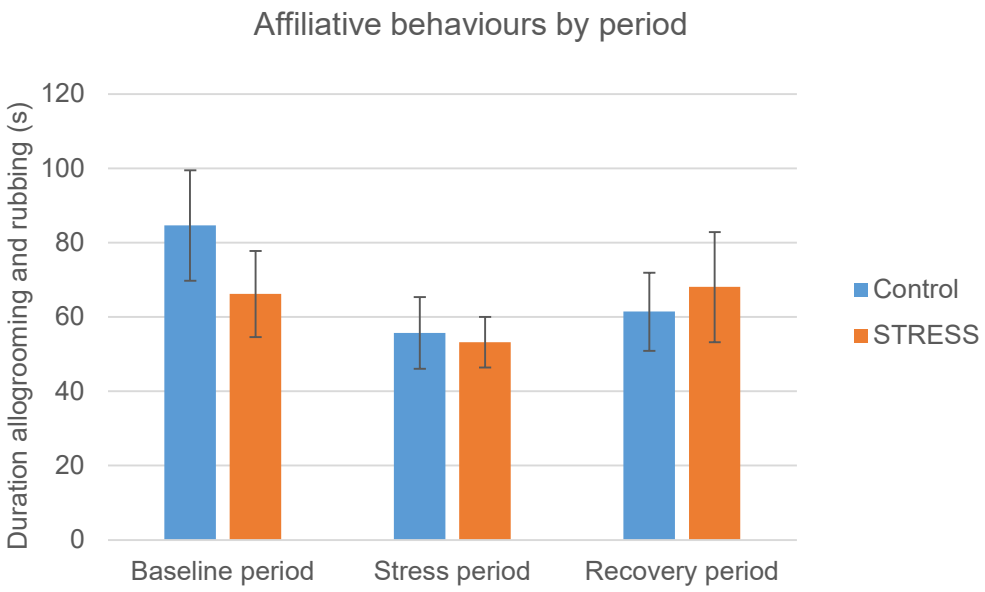


Figure 3.20. Average duration of observed affiliative behaviours by period. There were no significant differences in affiliative behaviour over time. Values represent mean ± SEM.

3.3.7 Attention bias test

Video observations for three animals had to be discarded, the first due to an error in the timing of the person entering the arena. The other two animals could not be evaluated because they attempted to jump over the fence, triggering the humane endpoints for the test leading to an early release from the arena.

3.3.7.1 Running

The total duration of running did not differ between the treatment groups. The only differences were due to timepoint ($F_{1, 80.2}$=20.32, p<0.001) whereby both groups showed very little running pre-startle but a significant increase post-startle (see Figure 3.21).

3.3.7.2 Walking

The total duration of walking was unaffected by treatment, but significantly increased post-startle ($F_{1, 69.2}$=5.00, p=0.029) (see Figure 3.22). There was also a significant sire effect on walking ($F_{9, 53.4}$=2.17, p=0.039).

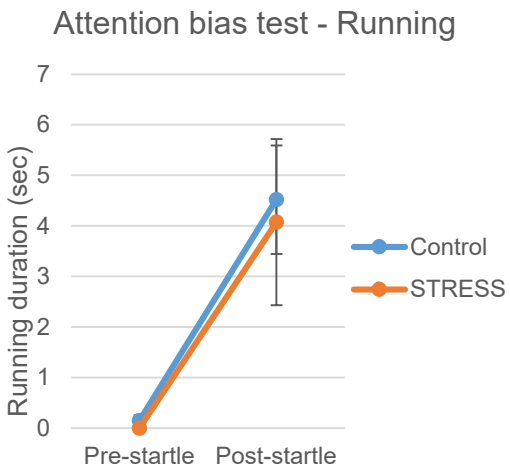


Figure 3.21. Running behaviour during the attention bias test. There was a significant increase in running post-startle but no differences between the treatment groups. Values represent mean ± SEM.

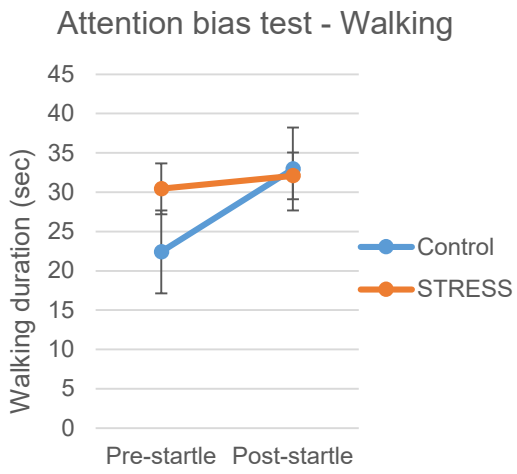


Figure 3.22. Walking behaviour during the attention bias test. There was a significant increase in walking post-startle but no differences between the groups. Values represent mean ± SEM.

3.3.7.3 Standing

Control animals spent more time standing than STRESS animals (33.91 vs 24.78 SED 3.252 sec; $F_{1, 17.2}$=10.42, p=0.005). There was a difference between the timepoints ($F_{1, 78.3}$=5.89, p=0.018), with a significant decrease in standing post-startle (see Figure

3.23). This was mainly due to an interaction between treatment and timepoint ($F_{1, 78.3}$=4.10, p=0.046), where control animals showed a significant reduction in time standing during the post-startle phase (39.44 pre-startle vs 28.38 post-startle SED 3.51 sec), whilst STRESS animals remained at their significantly lower level of standing throughout (25.23 pre-startle vs 24.23 post-startle SED 3.44 sec).

The other factor with a significant effect on standing was breed ($F_{1, 79.9}$=11.77, p<0.001), with AAx animals spending more time standing than LIMx (32.65 vs 26.04 SED 2.63 sec).

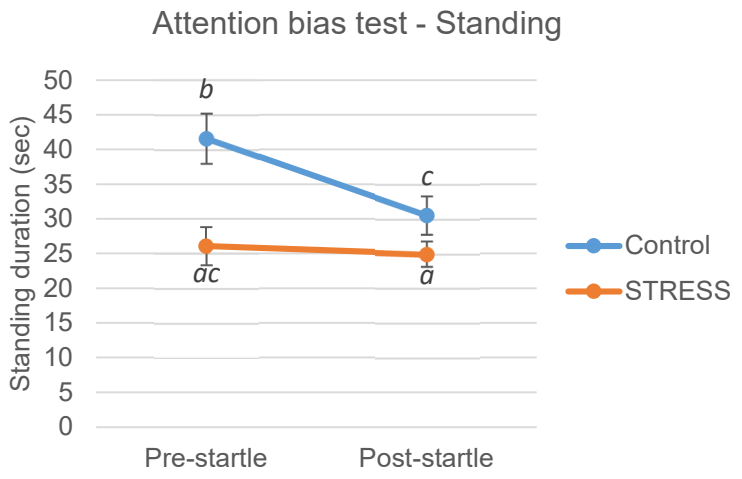


Figure 3.23. Standing behaviour during the attention bias test. There was a significant difference between treatments in standing pre-startle and an interaction between treatment and time. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

3.3.7.4 Vigilance

STRESS animals showed a tendency to spend more time being vigilant than control animals (32.68 vs 22.00 SED 3.566 sec; $F_{1, 76}$=3.90, p=0.052) but there was no effect of time (see Figure 3.24a). Vigilance pre-startle was significantly higher in the STRESS animals ($F_{1, 26}$=7.88, p=0.01; Figure 3.24b). Other factors also affecting vigilance were sire ($F_{9, 76}$=3.33, p=0.002) and CS ($F_{1, 76}$=7.16, p=0.009) which showed a significant negative effect on vigilance.

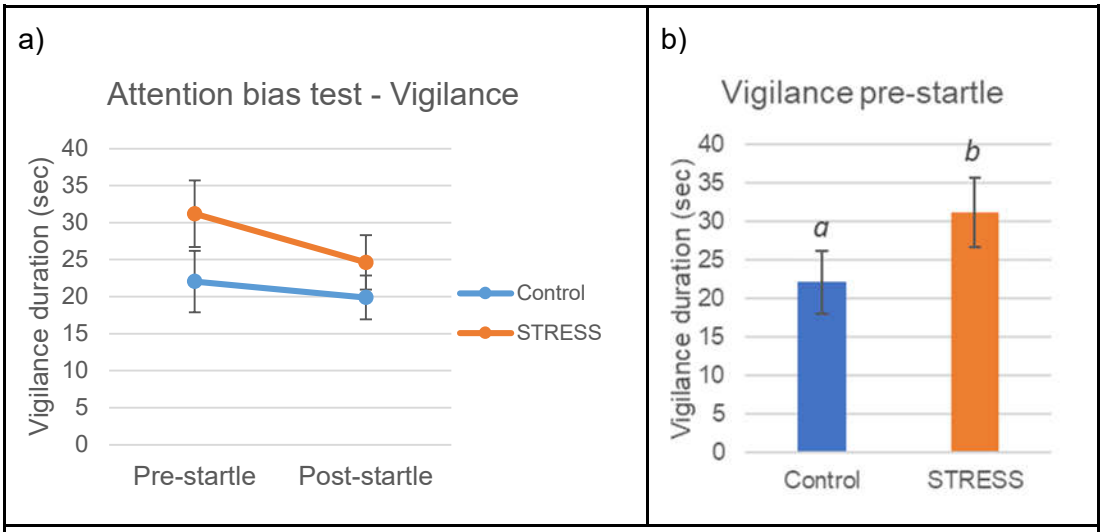


Figure 3.24. Vigilance behaviour during the attention bias test. (a) There was no significant change in vigilance over time (b) Vigilance pre-startle was significantly higher in the STRESS treatment animals. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

3.3.7.5 Immobility

Immobility decreased significantly post-startle (pre-startle 58.00 vs post-startle 47.62 SED 2.802 sec; $F_{1, 69.2}$=13.72, p<0.001). Other factors with an effect on immobility were sire ($F_{9, 69.4}$=3.58, p<0.001) and a negative effect of CS on immobility ($F_{1, 73.8}$=11.28, p<0.001) (Figure 3.25).

3.3.7.6 Area Crosses

Treatment had no effect on the number of areas crossed (Figure 3.26). However, there was a difference between timepoints ($F_{1, 68.9}$=19.90, p=<0.001) whereby area crosses increased significantly post-startle (13.81 vs pre-startle 7.99 SED 1.291). In addition, a statistical tendency for an interaction of timepoint with treatment was found ($F_{2, 33.8}$=2.58, p= 0.091). Control animals showed a significant increase in area crosses in the post-startle stage (pre- vs post-startle: 6.44 vs 15.22 LSD 3.751) in comparison to the STRESS group (9.53 vs 12.40 LSD 3.669). Other factors explaining the number of area crosses included sire ($F_{8, 71.4}$=2.89, p=0.008), breed (LIMx greater than AAx;

12.63 vs 9.16 SED 2.956; $F_{1, 71.3}$=3.44, p=0.068) and a positive effect of CS ($F_{1, 72.2}$=4.63, p=0.035).

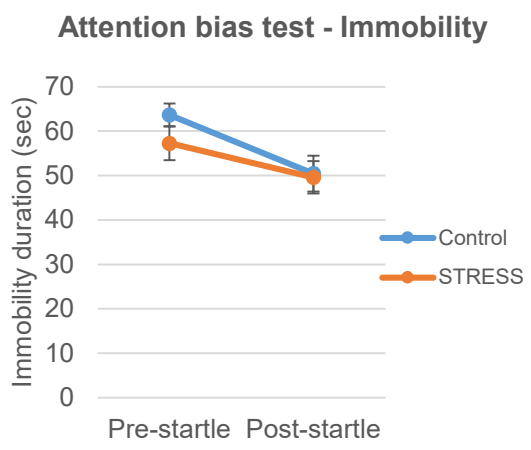


Figure 3.25. Immobility during the attention bias test. There was a significant reduction in immobility post-startle but no difference between treatments. Values represent mean ± SEM.

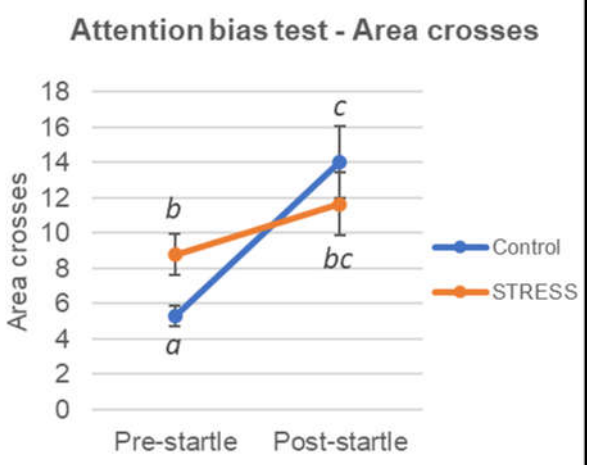


Figure 3.26. Number of area crosses during the attention bias test. There was a tendency for an increase in area crosses post-startle. Area crosses significantly increased in the control group post-startle. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

3.3.7.6 Looking at novel object

STRESS animals showed more attention to the novel object (curtain) than control animals (36.66 vs 20.64 SED 6.38 sec; $F_{1, 10}$=9.80, p=0.011; Figure 3.27a). There was also a statistical tendency for LIMx animals to pay more attention to the novel object than their AAx counterparts (34.8 vs 23.06 SED 6.66 sec; $F_{1, 38.4}$=3.64, p=0.064). CS ($F_{1,39}$=11.87, p<0.001) showed a negative effect on looking at the novel object. No treatment effect was found on the time spent looking at the startling stimulus (umbrella) (see Figure 3.27b), only effects of sire ($F_{9,}$29.1=2.35, p=0.04).

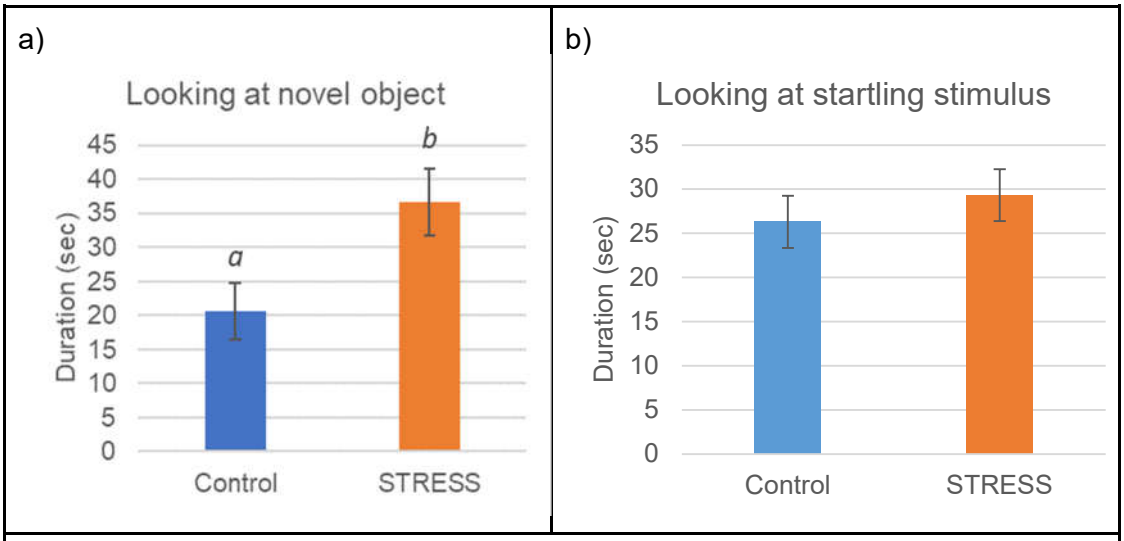


Figure 3.27. Attention to novel object (curtain) and looking at startling stimulus (person holding umbrella). (a) There was a significant difference between treatment groups in looking at the novel object (a) but no difference in looking at the startling stimulus (b). Values represent mean ± SEM. Means with different letters (a,b) are significantly different (p<0.05).

3.3.8 Feed intake

There were differences in dry matter intake (DMI) attributable to period ($F_{2,172}$=27.92.02, p<0.001) with DMI increasing over time, but no effects of treatment or interaction between treatment and period (see Figure 3.28). Part of the variation in DMI was explained by sire ($F_{8,172}$=3.24, p=0.002) and breed effects ($F_{1,172}$=12.02, p<0.001), in which AAx had a higher DMI than LIMx animals (11.00 vs 9.81 SED 0.32 kg/day). Similarly, temperament parameters also affected DMI. CS showed a significant effect ($F_{1,172}$=6.34, p=0.013) and FS a statistical tendency ($F_{1,172}$=3.57, p=0.06), where both higher CS and faster FS led to reduced intake.

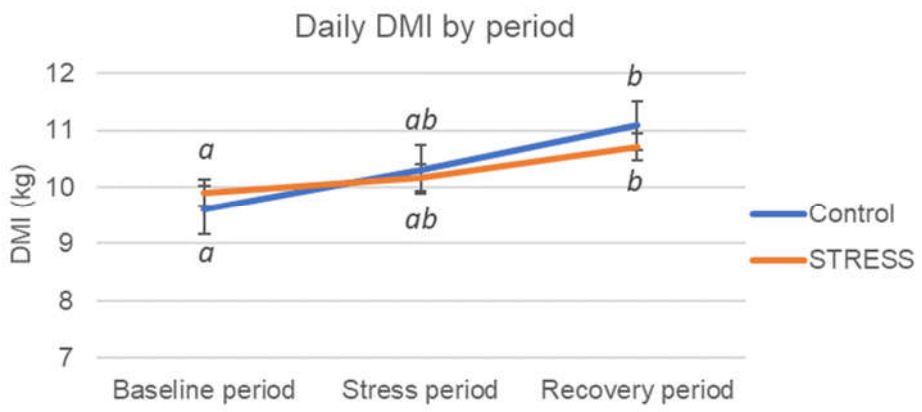


Figure 3.28. Average daily dry matter intake (DMI) by periods of the experiment and by treatment groups. DMI changed over time but was not affected by treatment. Values represent mean ± SEM. Means with different letters (a,b) are significantly different (p<0.05).

3.4 Discussion

In our experiment, we used the mixture of reduced space allowance as a continuous stressor, together with mixing, transport and isolation as acute stressors that happened weekly. Although none of the stressors was extreme on its own, it was expected that the mixture of stressors would combine to create what Ladewig (2000) describes as a chronic intermittent stressor. The results showed that cortisol differed between the treatment groups, but these differences were present before the stress period, whilst the ACTH test failed to find differences in adrenal sensitivity between the treatments. Locomotor activity was unaffected by the stress period except with regard to Motion Index, which fell at this time in the STRESS group. In the attention bias test, STRESS animals showed increased vigilance pre-startle and showed less locomotion than control animals after being startled, as indicated by the number of areas crossed. Furthermore, although the STRESS animals looked at the novel object (curtain) more than control animals, they did not look at the startling stimulus (umbrella) any more. There were no effects of the composite stressor treatment on agonistic or affiliative behaviour.

3.4.1 HPA axis response

As described by Gupta et al. (2004), a key feature that defines the stress response is the activation of the HPA axis. Plasma cortisol is metabolised rather quickly (Andrew et al., 2017). Studies in cattle report a 10-minute lag between the presentation of the stressor and peak plasma cortisol (Willett and Erb, 1972; Hernandez et al., 2014), making it a good measure of short-term changes in cortisol in response to a stressor. On the other hand, faecal cortisol metabolites reflect the circulating cortisol concentration 10 – 12 hours prior to sampling (Möstl et al., 2002). Therefore, they provide a useful measure to compare changes in baseline cortisol over time, whilst

avoiding the confounding effects of any short-term changes in plasma cortisol due to handling (Krawczel et al., 2012).

Additionally, faecal cortisol can be sampled more regularly as it is not as invasive as blood collection. In our case, faecal cortisol was assessed every two weeks; therefore, we had more comparison points than with plasma cortisol. Consequently, both measures could be used in a complementary manner. In the current experiment, the results from both the plasma and faecal cortisol did not show any significant effects in response to the composite stressor treatment. No previous experiments have used the same chronic intermittent stress regime used here, but studies using similar but separate stressors have found mixed effects on cortisol, as discussed subsequently.

Experiments using a reduced space allowance as a source of stress in cattle have resulted in diverse effects on basal cortisol. Previous studies have found that overcrowding due to changes in lying space that affected lying time resulted in increased plasma cortisol concentrations in dairy cows (Friend et al., 1979; González et al., 2003). Some authors have expressed that the most stressing element of higher stocking density is the competition for feeding space and space to lie down (Munksgaard and Simonsen, 1996). Therefore, it is possible that effects of stress from reduce floor space are manifest most readily when opportunities to feed and lie down are disturbed. In our experiment, floor space was reduced without affecting the animal to feeder ratio.

On the other hand, other studies have failed to evidence any effects on basal cortisol. For example, experiments by Huzzey et al. (2012) found no changes in basal plasma cortisol in response to overstocking in dairy cows (50% reduction in floor space), while Krawczel et al. (2012) found similar results using faecal cortisol (42% reduction in

space). Similarly, Silva et al. (2016) found no differences in plasma or hair cortisol between dairy cows with different stocking densities based on the availability of lying stalls (80 vs 100%). Specifically, with beef heifers, Fisher et al. (1997a) found no effect of stocking densities ranging from 1.5 to 3 m^2 per animal on slatted floors on basal plasma cortisol or cortisol in response to an ACTH challenge. Our space allowance, although reduced from 8.72m^2 to 4.35m^2 per animal, might not have been restrictive enough as it did not affect lying time. In our experiment, feeder availability was constant given that what changed was the size of the pen during the stress period rather than the number of animals per pen, perhaps facilitating the adaptation to a smaller space.

Hickey et al. (2003) evaluated the effects on welfare of space allowances ranging from 1.5 to 4 m^2 per finishing steer on slatted floors. Parameters evaluated included behaviour (lying, eating and social interactions), productivity, cleanliness scores, haematological parameters (cell counts, acute phase proteins, fibrinogen) and blood chemistry parameters. However, this study did not analyse cortisol levels. Adverse effects on the parameters studied were found only when space allowance was under 3 m^2 per animal. No differences in haematological or metabolic parameters were found in a subsequent study (Keane et al. 2017) using finishing beef heifers at space allowances ranging from 3 to 6 m^2 on slatted floors. This information taken together could indicate that the levels of space allowance we used, although below the minimum recommendations (as per British Standard for Livestock Buildings BS 5502–40(2005)), might not have been restrictive enough to induce chronic stress on its own.

In regards to stress responses to transport, authors have reported that the peak in HPA axis response occurs during the loading process rather than actual

transportation (Burdick et al., 2011b), with plasma cortisol levels returning to normal hours after transport (Knights and Smith, 2007; Buckham Sporer et al., 2008; Kang et al., 2017). In the case of faecal cortisol, it is found to increase approximately 12 hours after loading for transport (Palme et al., 2000; Möstl et al., 2002) with values returning to normal one day post-transport (Palme et al., 2000). Therefore, transport causes an acute transient increase in cortisol in cattle (Tarrant et al., 1992; Knights and Smith, 2007; Earley et al., 2012), but some authors suggest that the duration of the transport is not a crucial factor in transport stress (Sartorelli et al., 1992), and instead novelty and loading are probably the most stressful elements of the transport process (Broom et al., 1996; Hall et al., 1998). Therefore, it is likely that the transport events used in our experiment, although being short in duration, would be expected to cause repeated stress since the loading and unloading are the most challenging components of a journey.

There have been fewer experiments with repeated transport; however, a common finding in such studies is that cattle tend to habituate to repeated transport stress. For example, Locatelli et al. (1989) found that in calves subjected to repeated transport for 30 minutes, the increase in cortisol became less marked in successive trials. Similarly, Lay et al. (1996) found that cows exposed to repeated transport reduced their cortisol responses as early as the third transportation event, showing desensitisation to the transport process. It is therefore possible the steers from the STRESS group could have habituated to transportation quite early in the process as they got accustomed to loading and unloading. It is known that for events that are not novel, the predictability and perceived control over the situation can reduce considerably the stressfulness of the event (Koolhaas et al., 2011).

Social isolation as a cause for acute adrenocortical responses, has been well described in dairy cattle (Rushen et al., 1999, 2001). Some authors have found that

short periods of social isolation (15 min) led to acute increases in plasma cortisol and reduced nociception (Rushen et al., 1999; Herskin and Munksgaard, 2004; Herskin et al., 2007) in dairy cattle. Similarly, in beef cattle, acute changes in cortisol were seen in response to 8 minutes of isolation from pen mates (Boissy and Le Neindre, 1997), and cortisol has been found to remain elevated during the entirety of a two-hour isolation test (Ninomiya and Sato, 2011). Social isolation does appear to be an effective acute stressor. For example, Herskin et al. (2007) found a much larger adrenocorticotropic response to 15 minutes isolation than to 15 minutes of restraint. However, even though isolation is an effective stressor, it is still subject to habituation. Schrader and Müller (2005) reported that dairy cows showed a blunted cortisol response over four exposures to a 20-minute isolation test. In our study, we did not measure specific responses during the isolation procedures. However, although novel stimuli were added to reduce habituation, the effects of ten-minute isolation episodes were probably short-lived, particularly towards the end of the 8-week stress period.

Mixing of unfamiliar cattle leads to aggression and sexual interactions in males, especially in groups of animals of similar body size and weight (Mounier et al., 2006). This temporary increase in aggression due to mixing might not necessarily translate into increased basal cortisol levels or HPA axis responsiveness (Veissier et al., 2001; Mounier et al., 2005). Additionally, mixing might not affect all animals in the group equally. For example, in beef cattle, Mench et al. (1990) only found increased basal cortisol in response to mixing in the socially subordinate individuals. When new animals are introduced into an already established group, it is common that greater aggression is directed at the alien cattle (Mench et al., 1990). In our study, this could have created different levels of stress for our STRESS Rotate animals. However, we only assessed physiological and behavioural parameters on the resident steers

(STRESS Stay), which may have been at an advantage when mixing occurred, as residents were a larger group and in their familiar pen, reducing the effects of mixing.

Considering the information on mixing, reduced space allowance, transport and isolation as individual stressors, the evidence suggests that, for most, they do induce acute activation of the HPA axis but do not readily impact basal cortisol levels. In the experience of the present experiment, these same stressors used in combination (at least at the levels included in this trial) do not constitute a sufficient chronic intermittent stress regime to affect basal cortisol levels. This finding could have potential implications for beef cattle husbandry practices and animal welfare, a subject that will be addressed later in this discussion.

ACTH challenge tests have been used as a standard research methodology for confirming changes in adrenal sensitivity as a consequence of chronic stress. In our experiment, there was a significant increase in cortisol 30 and 60 minutes after the injection of Synacthen, which as expected confirms the ACTH-mimicking-drug induced cortisol release successfully from the adrenal glands. However, there was no difference in response between treatments confirming that the STRESS group did not show any changes in adrenal sensitivity.

In the literature, results from an ACTH challenge due to chronic stressors are varied and inconsistent. Some authors have found augmented cortisol due to adrenal sensitisation to ACTH in weekly-regrouped calves (Veissier et al., 2001), cows mixed at a high stocking density (Friend et al., 1977) and overcrowding in the resting area (Friend et al., 1979). On the contrary, other authors have found the opposite effect with a reduction in cortisol responses after an ACTH challenge, pointing to reduced adrenal sensitivity as a result of long-term over-exposure to endogenous cortisol. This is the case for several studies using social isolation (van Reenen et al., 2000) and

weekly regrouping (Raussi et al., 2004) in calves, tethered bulls (Ladewig and Smidt, 1989) and restricted space in beef cattle (Fisher et al., 1997a, 1997b). A critical review performed by Dickens and Romero (2013) evaluating chronic stress studies in captive and wild animals (including laboratory species) attempted to find a consensus on the endocrine profile of chronically stressed wild animals. These authors concluded that, given the vast variation in stress responses reported in the literature, a consistent and predictable endocrine response to chronic stress does not exist. Therefore, they advocate that when assessing chronic stress, the important issue is to evidence those changes happening in glucocorticoid regulation, however, the direction of such change (increase, decrease or temporal change) may be relatively less important than identifying that any change in stress responses is happening at all.

Another possible explanation for these conflicting results may lie in the mechanisms involved in adapting to a stressor, set to prevent prolonged exposure to elevated cortisol concentrations. Following this rationale, some authors suggest that adrenal responsiveness can increase in the short term under stressful conditions in cattle, leading to higher cortisol in response to ACTH. However, more prolonged exposure to the stressor may decrease adrenal sensitivity, leading to reduced production of glucocorticoids in response to ACTH (Mormède et al., 2007). For example, Friend et al. (1977), using crowding stress in dairy cows, found that 2 and 3 days of treatment led to increased ACTH test cortisol responses, whereas 9 days of crowding led to reduced sensitivity to ACTH. Similarly, Munksgaard et al. (1999) also found a change in HPA axis response as the treatment progressed in tethered bulls prevented from lying down 14 hours a day for 10 weeks. Other authors have found that individual factors may also play a role in these changes in HPA axis sensitivity. For example, Hasegawa et al. (1997) found that social mixing increased cortisol in response to an ACTH challenge only in the higher-ranking heifers. Therefore, group

comparisons made without taking individual factors into consideration might mask differences. Normal levels of cortisol or ACTH responses do not necessarily mean the animal has habituated centrally to the stressor, or that there was no chronic stress response (Smith and Dobson, 2002; Knights and Smith, 2007). For example, some authors report behavioural adaptations to stressors in the absence of any changes in the HPA axis response (Munksgaard and Simonsen, 1996; Chen et al., 2015). Therefore, assessing behavioural responses is also necessary in order to evidence other effects of chronic stress. As has been stated in the past, there is no "gold standard" biomarker to diagnose chronic stress reliably (Ladewig, 2000; Lee et al., 2015). Glucocorticoid secretion during a stress response serves to regulate the whole-body energy homeostasis (Sapolsky et al., 2000) and, as such, it is highly unspecific. Therefore, glucocorticoid responses need to be viewed in context. It is only possible to infer the existence of chronic stress by assessing multiple physiological and behavioural parameters, as such further behavioural changes in response to stress were assessed.

3.4.2 Locomotor activity

In our experiment, we did not find any effects of the composite stressor treatment on daily lying duration, average lying bout duration or average standing bout duration. The stressor that appeared to be the most obvious candidate to disrupt lying time would be space allowance. Studies have reported that smaller space allowances decrease lying time in beef (Fisher et al., 1997a) and dairy cattle (Krawczel et al., 2012; Telezhenko et al., 2012). However, other studies found no differences in lying behaviour in dairy cattle in response to different stocking densities (Collings et al., 2011; Huzzey et al., 2012). Similarly, a study by Fustini et al. (2017) in dairy cattle that used a space allowance of 12 m^2 versus 4.8 m^2, which are comparable to those

we used (8.72m^2 vs 4.35m^2), also found no difference in lying behaviour between the groups.

Motion Index was the only activity parameter that was influenced by the interaction of treatment and period, being significantly lower for STRESS animals during the Stress period. Since Motion Index expresses the overall activity of the steer based on acceleration (Kokin et al., 2014), the result indicates that STRESS animals showed fewer movements with rapid acceleration. Although states of chronic stress could induce slower movements, given that all other activity parameters were unaffected by the stress treatment, the more likely explanation is that the smaller pen size available during the stress period was insufficient to allow high acceleration movements such as running. Therefore, the physical limitation of movement might have affected the Motion Index more than other activity parameters.

In this study, temperament showed an effect on various locomotor activity parameters. Animals with higher flight speed showed less lying time, longer standing bouts and higher Motion Index, whilst higher crush score led to more steps per day and higher Motion Index. Together, these results suggest that more temperamental cattle were more active in their home pen. Previous studies have also found correlations between activity parameters and temperament in cattle. Cziszter et al. (2016) found that dual-purpose cows of more nervous temperament performed more steps per day, whilst MacKay et al. (2013) found that higher FS predicted higher Motion Index and average daily step count in beef cattle. Hence, our results concur with those in the literature.

3.4.3 Home pen social behaviour

There were no major changes in the agonistic and affiliative social behaviours in the home pen in response to the composite stressor treatment. Social mixing can

increase agonistic behaviours (Mounier et al., 2006), but in our study, we found no evidence of increased agonistic behaviour in the STRESS group in comparison to the control group. Studies have reported that much of the fighting happens during the first few hours after mixing, declining rapidly within the next few days (Fraser and Rushen, 1987). It is likely that there was a transient increase in aggression in the STRESS group soon after mixing, which may have decreased by the time observations were made on days that animals were not handled. Furthermore, mixing was performed by keeping six resident animals in their home pen (STRESS Stay) and introducing four STRESS Rotate animals from another pen. Alien cattle are seen as subordinates when introduced into an established herd and receive most aggression (Mench et al., 1990). It is possible that greater aggression was directed to the STRESS Rotate animals, which were not included in the analysis.

Reduced space allowance may lead to greater aggression during feeding (Hasegawa et al., 1997; Collings et al., 2011; Lobeck-Luchterhand et al., 2015). However, other authors concur with our results that reducing floor space allowance has no impact on aggression when feeding (Telezhenko et al., 2012). In our experiment, the way space was reduced in STRESS pens did not affect the feeder space or *ad libitum* feed availability. Therefore, it is likely that competition for access to feed was similar in the STRESS and control groups.

Agonistic interactions for both treatments decreased over time. It is known that aggression tends to decrease once dominance hierarchies become established (Grant and Albright, 2000). Retaliations were not affected by treatment or period as main effects. However, retaliations did significantly increase in the STRESS group during the stress period before declining again in the recovery period, whereas retaliations remained the same for control animals. It is possible that the composite stressor treatment created less stable dominance relationships, leading to an

increase in retaliations to aggression. However, it is noteworthy that there was no difference in the rate of retaliations between the control and STRESS animals during the stress period, suggesting that the STRESS animals behaved in a similar way to the control animals in absolute terms.

There was a low occurrence of both social rubbing and social licking, and so, these were grouped as affiliative behaviours for analysis. The observation period used to assess these behaviours may have been too short to record them individually, but other authors have also reported a low rate of occurrence of affiliative behaviours (Aménd ola et al., 2016). Affiliative behaviours did not show differences between treatments or periods in the study.

There is conflicting information regarding the effect stressful environments can have on affiliative behaviours. Some authors argue that more affiliative behaviours tend to occur in situations where other welfare needs are met and are positive for group cohesion (Sato et al., 1993). Conversely, others report that social rubbing and licking may be used to reduce tension in groups as appeasement behaviours after agonistic interactions (Boissy et al., 2007; Napolitano et al., 2009). Although the interpretation of affiliative behaviours on their own may be challenging, this study found no effect of the stress period on their occurrence and, like the agonistic interactions, it appears that the composite stressor treatment had little effect on the social behaviour in the home pen.

3.4.4 Attention bias test

The purpose of the attention bias test was to assess any treatment differences in behavioural responses that could suggest a more anxious state when exposed to a potential threat (Crump et al., 2018). Attention bias is a form of judgment bias that can be assayed rapidly without training, unlike more laborious cognitive bias testing (Lee

et al., 2016; Monk et al., 2018; Ede et al., 2019). Activity increased post-startle in both groups (increased running, walking and area crosses, and less immobility) without differences between the treatments. Although there was no significant difference between groups in the time spent immobile, what each group did while immobile was different. During the pre-startle stage, STRESS animals spent significantly more time being vigilant towards the novel object (curtain) suggesting a possible attention bias to this new unknown stimulus. However, during the post-startle stage, there was no difference between groups in their attention to the startling stimulus (umbrella). The heightened pre-startle attention may be compatible with differences in affective state leading to an attention bias, but if so, it would be expected that STRESS animals would also show heightened attention to the umbrella.

An alternative explanation is that both groups had similar anxiety, but previous experience of isolation might have affected the initial stage of the test. STRESS animals were more used to being isolated and may have focused on the novel object, whereas the control animals may have spent this time looking for an escape to return to the herd.

It is also possible that the animals were in different anxiety states, but the methodology employed did not allow us to detect more subtle differences in response to the potential threat. For example, it could be that the startling stimulus was too abrupt and aversive so that fear responses took over and both groups focussed on escaping, reducing the time available to look at the threat as an ambiguous stimulus. Other authors have used a threatening stimulus for a short period of 10-15 seconds before removing it, and then assessing the attention paid towards where the stimulus had been (Lee et al., 2016). Perhaps this would have rendered different results to the post-startle stage.

It was interesting to see temperament effects on the test. For example, higher CS resulted in animals moving more during the test (area crosses), probably while looking for a way out of the arena. Consequently, these animals spent less time being immobile, vigilant, and looking at the novel object. The CS has previously been shown to correlate with response to isolation (Turner et al., 2011a, 2013). Interesting FS did not affect any parameters of the attention bias test, perhaps confirming that CS and FS measure different underlying elements of temperament as described in earlier work (Turner et al., 2011a). However, the effects of CS do show that responses to the attention bias test might also be influenced by temperament and personality traits. This alone may warrant further study in the future.

These results indicate few differences in behavioural responses and attention to a novel stimulus by animals subjected to a composite stressor treatment. Future studies with fattening cattle ought to consider using a less startling stimulus and presenting it for only a short period before removing it.

3.4.5 Feed intake

As expected, DMI increased over time as the animals grew. There was no effect of the composite stressor treatment on DMI. Much of the literature on the relationship between stress and feed intake focuses on thermal stress, which, due to its different physiological effects, is not applicable to our model system. There are reports of particular sources of stress causing a transient reduction in DMI. For example, Burdizzo castration can cause a reduction in DMI for up to 10 days post-procedure (Fisher et al., 1996) and cattle reduce intake due to isolation stress (Llonch et al., 2018b).

Similarly, reports of decreased intake are not uncommon in response to overstocking (Collings et al., 2011). However, this is likely to be an effect of increased competition

at the feeders. Others have found no effects of stress on DMI. Gupta et al. (2008) found no effect of repeated regrouping and relocation on the DMI of steers, and others suggest that mixing has only a short-lived effect on intake for a few days post regrouping (Grant and Albright, 2001; von Keyserlingk et al., 2008; Schirmann et al., 2011).

However, it is generally agreed that responses to stress temporarily inhibit non-essential functions (e.g. growth and reproduction) in order to focus resources on survival (Ladewig, 2000). Therefore, it ought to be expected that different sources and severities of stress will have different effects on feed intake depending on the needs of the animal. Response to some stressors may demand a decrease in intake, whilst others require preservation of normal intake levels or an increase in appetite.

In our experiment, more temperamental animals (higher CS and faster FS) showed reduced intake. This is not an uncommon finding as other authors have reported reduced intake in relation to faster FS (Hoppe et al., 2010; Cafe et al., 2011) and both FS and CS (Petherick et al., 2003). It is interesting to note that this finding coincides with other studies that report slower weight gain in temperamental animals (Voisinet et al., 1997; Reinhardt et al., 2009; Turner et al., 2011b). In the future, it might be interesting to assess the mechanisms by which temperament impacts intake, growth and activity.

3.4.6 Resilience to commercial stressors

Steers in this trial were subjected to multiple stressors at levels beyond what would be expected on a commercial farm. However, we were not able to identify obvious effects of chronic stress in response to the treatment. Continuous activation of stress responses is detrimental for the organism; hence animals tend to adapt to stressors, especially those that can be predicted. Examples in the literature are common,

showing that cattle will reduce their responsiveness to repeated restraint (Andrade et al., 2001), transport (Price et al., 2015) and isolation (Schrader and Müller, 2005) to name a few. Similar reports show that sheep can habituate to isolation, transport and other repeated stressors as well (Coppinger et al., 1991; Cockram et al., 1994; Hall et al., 1998; Roussel et al., 2004,2006; Wickham et al., 2012).

It is possible that the steers in our experiment showed resilience to the applied stressors. Colditz and Hine (2016) define resilience as the animal's capacity to be minimally affected by a disturbance or to adapt rapidly in order to return to the physiological, behavioural, health, affective and production states before the disturbance occurred. This process can occur at many levels, but most likely, it will involve the development of a reduction in the sensitivity to the stimulus, in addition to adaptive neurophysiological responses and behavioural changes (Russo et al., 2012; Galán et al., 2018). However, not all animals experience stressors in the same way, and there will be variability in coping strategies and level of resilience. Only when multiple acute stressors are sufficient to deplete reserves and affect other biological functions would this lead to obvious distress. We were not able to produce a chronic stress state explicitly in the STRESS group. However, given these individual differences in response, individual animals might have experienced a chronic stress response but, since chronic stress can be manifest in different ways, this was not picked up at the group level. In future work, it would be interesting to assess these individual differences in resilience and stress responsiveness in more detail to understand chronic stress in greater depth.

3.5 Conclusion

The composite stressor treatment used in this experiment, based on a reduced space allowance as a continuous stressor, together with mixing, transport and isolation as

acute stressors on a weekly basis, was expected to create a chronic intermittent stress. The results showed that cortisol differed between the treatment groups, but these differences could not be attributed to the treatment, whilst the ACTH test failed to find differences in adrenal sensitivity between the treatments. Motion Index was the only parameter that showed differences between the groups. There were no effects of the composite stressor treatment on agonistic or affiliative behaviour. In the attention bias test, STRESS animals were more vigilant pre-startle and showed less locomotion than control animals after being startled, as indicated by the number of areas crossed. Additionally, STRESS animals looked at the novel object (curtain) more than control animals. However, no differences were found in attention to the startling stimulus (umbrella).

Although it was not possible to find clear evidence of chronic stress, the results indicate that beef cattle show a degree of resilience to repeated but predictable stressors, from a behavioural and stress physiology point of view. However, this study did not assess the welfare implications of the applied treatment. Although this research furthers our understanding of the resilience of cattle to multiple common commercially relevant stressors at levels beyond what would be expected on a typical farm, the mechanisms by which this resilience is achieved would be worth investigating, as would the level of repeated acute stressors needed to develop evident chronic stress in beef cattle. In the following chapter, the effects of the stress regime imposed in this study on feed efficiency, the microbiome and methane emissions are explored.

Chapter 4 - The effects of a composite stress treatment on individual productivity, the rumen microbiota and methane emissions

4.1 Introduction

The rumen microbiome comprises a complex network of microorganisms essential to the biology of its ruminant host. As such, the disruption of the normal balance of microbiota can have a major effect on the health, welfare and overall production efficiency of cattle (Jami and Mizrahi, 2012; Henderson et al., 2015; Malmuthuge et al., 2015). A large and growing body of literature has shown that prolonged stressors can drastically change the balance of gut microbiota in monogastrics, affecting immunity, susceptibility to pathogens or opportunistic bacteria (Bailey et al., 2010, 2011; Freestone and Lyte, 2010). However, studies on the impact of stress on the rumen microbiota are few. There have been a limited number of studies in cattle that have confirmed changes in the microbiome following short term stressors such as transport (Deng et al., 2017; Li et al., 2019) and a longer-term stressor such as heat stress (Uyeno et al., 2010; Chen et al., 2018; Baek et al., 2020). However, to the best of my knowledge, there is no available research into how repeated stressors can impact the normal rumen microbiome. This is important because disruption of the rumen microbiome could have important consequences for the suboptimal use of nutrients and could affect productivity and health. No study has investigated the effect of how multiple repeated commercially relevant stressors may impact the rumen microbiome in beef cattle.

This chapter will complement those results from Chapter 3 by assessing any changes in the rumen microbiome in response to a composite stressor treatment comprised of

a reduced space allowance in addition to weekly mixing, transport, and isolation. Besides evaluating the effects on productivity, a small cohort of animals from this trial was used to assess any effects of the composite stressor treatment on methane emissions.

Over the last decade, greenhouse gas (GHG) emissions from cattle have received much interest, given the substantial contribution of agriculture to human-produced GHG emissions. Depending on the estimate, agriculture is believed to contribute between 7 and 18% of total anthropogenic GHG emissions (Hristov et al., 2013). Enteric fermentation from ruminants is the largest source of livestock GHG, producing around 40% of agricultural emissions (Gerber et al., 2013b). Given its long production cycle and system inefficiency, beef cattle are the livestock with the highest emissions per unit of final product, needing 46.2 kg CO_2-eq per kg of carcass weight, which is more than 16 times the emissions per kg of milk (Opio et al., 2013). In order to reduce the methane emissions from beef cattle, the highest mitigation potential will come from reducing emissions per unit of meat produced and reducing sources of inefficiency in production.

Methane emissions produced by archaea living in the rumen of cattle are responsible for 30.8% of agricultural GHG emissions (Gerber et al., 2013b); ruminants producing aproximately 20% of global methane emissions (Hua et al., 2018). Methane not only has detrimental effects on the environment but also has productivity implications as ruminal methanogenesis represents an energy loss estimated to be as much as between 2 and 12% of potential gross energy from ingested feed (Johnson and Johnson, 1995; Conrad, 2009). This energy loss is because methanogenesis involves the reduction of a source of H_2 in addition to carbon dioxide to form methane (CH_4); resources that potentially could be used for more energy-efficient pathways such as propionate formation (Janssen, 2010; Gagen et al., 2015). Thus, knowledge of the

effects of repeated stressors on microbial communities and the production of methane could inform future methane mitigation strategies targeted to beef cattle.

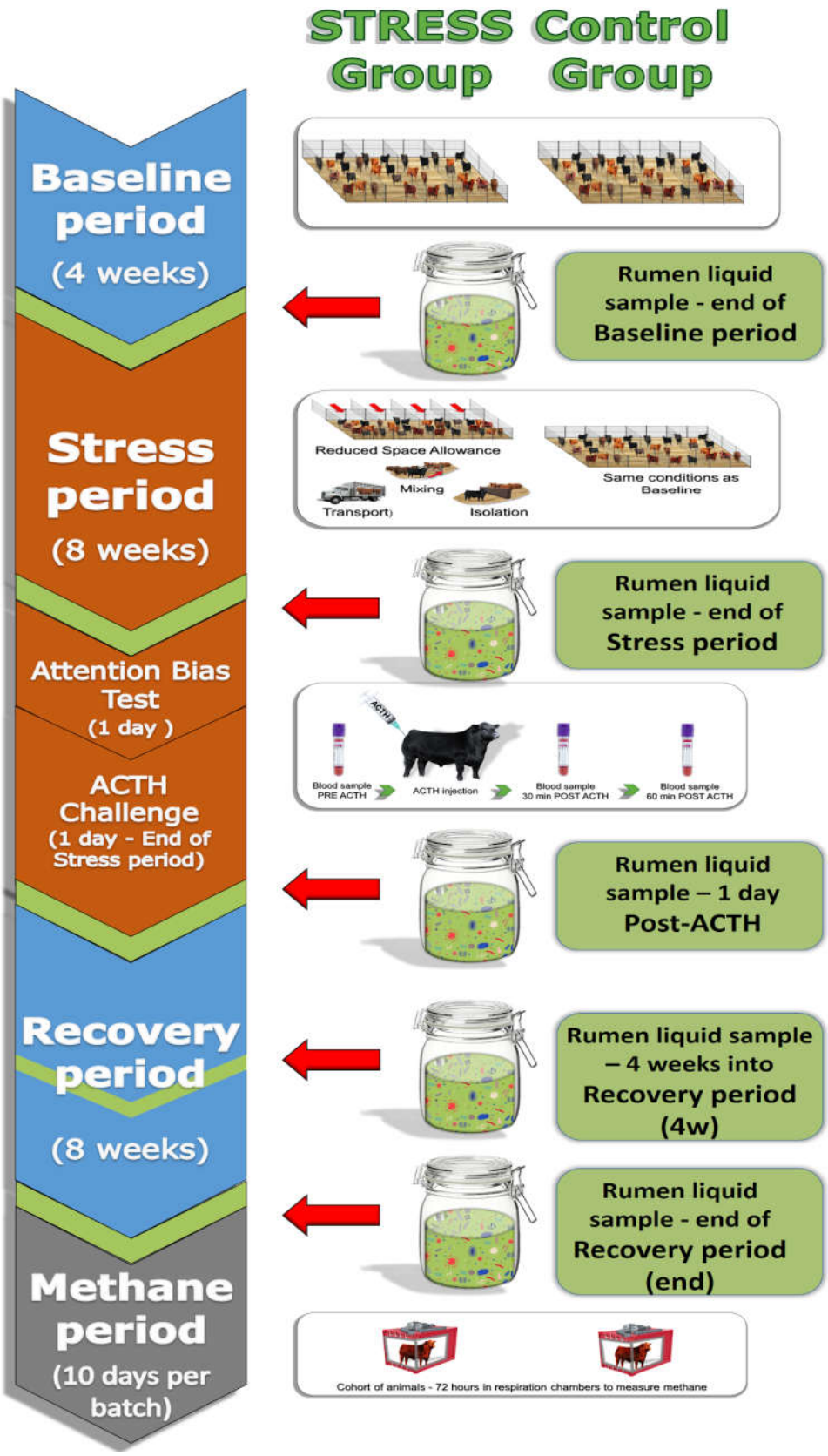
To complement the results presented in Chapter 3, and given the lack of research assessing the effect of repeated commercial stressors on the rumen microbial populations of beef cattle, the main aim of this chapter was to identify effects of a composite stressor treatment on the rumen microbiota and productivity, as well as a small trial to assess effects on methane emissions.

4.2 Materials and methods

4.2.1 Experimental design

The experimental design has been previously presented in Chapter 3. Here, the aspects relevant to the rumen microbiome analysis are described in detail. As described in the previous chapter, rumen samples were collected from the steers (n=63) throughout the experiment for metagenomic analysis. The sampling was performed using the same nasogastric intubation technique described in Chapter 2, and the samples were handled and preserved using the same protocol. Rumen contents samples were collected at five timepoints, the first sample collected at the end of the Baseline phase, to establish the state of the microbiome before the composite stressor treatment. A second rumen contents sample was collected at the end of the last week (week 8) of the Stress period to evaluate changes due to treatment. The third sampling time point occurred the day after the ACTH test was completed for each pen, i.e. once a pen was treated with ACTH, the following day in the afternoon, animals in that pen were sampled for rumen contents. This was termed the Post-ACTH timepoint. This timepoint was used to assess any changes in response to ACTH. Two more rumen contents samples were taken during the Recovery period, the first one at 4 weeks into the Recovery period (Recovery 4w) and the last one at the end of the Recovery period (Recovery end). These were all used to monitor longitudinal changes in microbiome diversity after the Stress treatment was completed. A schematic of the sampling timepoints can be found in Figure 4.1.

Figure 4.1. Schematic diagram showing rumen liquid sampling points in relation to the experimental phases.



DNA was extracted following the protocol in Chapter 2 and based on Yu and Morrison (2004), with the difference that repeated bead-beating followed by precipitation, elution and purification were performed using the QIAamp® DNA Stool Mini Kit, (QIAGEN, Dusseldorf, Germany). DNA extraction and amplicon library preparation were carried out in collaboration with the University of Aberdeen. PCR used barcoded universal prokaryotic primers targeting the V4 region of the 16S rRNA gene (Thompson et al., 2017) and Q5® High-Fidelity DNA polymerase (New England Biolabs Inc., Hitchin, UK). Twenty-five µL reactions were run in quadruplicate for 20 cycles.

PCR products were cleaned and quantitated using the Qubit high sensitivity dsDNA assay kit (Fisher Scientific UK Ltd., Loughborough, UK). The samples were pooled in equimolar quantities and 80 µL run on a 1% w/v agarose/TBE gel to remove primers and dNTPs. The band at the expected size containing the amplicons was cut and the DNA purified using a Wizard® SV Gel purification kit (Promega UK, Southampton, UK). The libraries were quality assessed using an Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA) and sequenced using an Illumina MiSeq with v2 250 paired-end reagent kits (Illumina UK, Cambridge, UK.).

4.2.2 Bioinformatics

Bioinformatics were performed by collaborators at the University of Aberdeen and following the analysis pipeline used by Snelling et al. (2019). The sequence data were analysed using mothur 1.39.0 (Schloss et al., 2009) with steps to assemble paired-end sequences, remove low-quality sequences using both quality control metrics and chimaera removal using UCHIME 4.2.40 (Edgar et al., 2011). Sequence counts in each library were normalised by sub-sampling to 20,000 sequences per sample. An operational taxonomic unit (OTU) based approach was selected with sequences

clustered into OTUs using OptiClust at 97% identity, singletons removed and taxonomic classification of the representative sequences using the Silva 128 reference database (Quast et al., 2012).

Microbial community data was tested for coverage per library using Good's statistic (Good, 1953). Microbial community species alpha diversity was assessed using the Shannon diversity index, whereas the beta diversity was calculated using the Bray-Curtis dissimilarity index (Bray and Curtis, 1957). Bray-Curtis dissimilarity is one of the most commonly used beta diversity metrics to quantify the compositional dissimilarity in species between two different sampling locations. It is a proportion calculated from the count of sightings of shared species in the two sites divided by the total number of observed species, which is then subtracted from one to assess how dissimilar the sites are. Therefore, the closer the Bray-Curtis is to 0, the more similar the sites; the closer to one, the fewer species they share. This metric is useful and straightforward when dealing with few sites. However, since in metagenomics we are dealing with many samples with a large number of species, this creates a vast matrix of pairwise measures of Bray-Curtis dissimilarity. Therefore, it is necessary to employ ordination techniques that facilitate handling the dimensionality of the data. In this case, in order to visualise clustering between individual samples by category (i.e. treatment, period and breed), non-linear multidimensional scaling (NMDS) was used. NMDS is an ordination technique that focuses on condensing multidimensional data into a smaller number of axes (usually just 2 or 3 dimensions) while preserving the rank order and underlying distances. Unlike Principal Component Analysis, it does not maximise the variance explained by the axes, as this leads to many dimensions that describe different amounts of variance. Therefore, NMDS is a useful tool in microbial ecology to condense and analyse multidimensional data. In our analysis, the NMDS coordinates for each sample were created based on the Bray Curtis

dissimilarity matrix. These were later inspected and visualised using Plotly in R for group comparisons.

Partial Least-Squares Discriminant Analysis (PLS-DA) is a multivariate dimensionality-reduction tool useful as a feature selector or classifier (Barker and Rayens, 2003). PLS-DA is a powerful methodology in situations where there are many more features (variables) than observations (samples), as is the case in the present study with many OTUs per sample. This model is able to discriminate between groups of samples (i.e. categories) by rotating PCA (Principal Components Analysis) components to determine those variables responsible for the maximum separation between the sample groups and that predict these categories. Those variables that have the greatest contribution to the PLS-DA are usually referred to as the 'variables important in projection '(VIP). Therefore, the VIP score is a measure that summarises the contribution of each variable to the PLS-DA model, allowing the ranking of variables according to their predictive importance. For this experiment, a PLS-DA was performed at OTU level to determine compositional features that were important to discriminate between STRESS and Control groups during the Stress period. The PLS-DA regression was run with treatment as the Y response variable, OTU as the predictor X variables and breed as a fixed effect. The output was set to two PLS-DA components. The resulting regression was rerun to predict the treatment groups based on the OTU dataset and determine the R^2 of the prediction. OTUs with a VIP greater than 1 were used for additional analysis.

In order to be able to perform a more descriptive comparison of any OTU changes over treatments and time, a linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) approach was used. LEfSe is a tool that identifies taxonomic features (e.g. OTU) most likely to explain differences between groups. LEfSe uses a multistep process, first using Kruskal-Wallis tests to detect features with significant

differential abundance with respect to the explanatory category. Then, pairwise comparisons are run between subclasses using the unpaired Wilcoxon rank-sum test to find those features with more likely biological significance. As a third step, LEfSe uses Linear Discriminant Analysis to estimate the effect size of each differentially abundant feature. LEfSe is particularly useful as it can estimate the magnitude of the effect size of those differences in features between groups (i.e. LDA Score), making it a valuable tool to rank different features and find those most associated with different groups. In this study, we used an LDA score of over 3 to determine those taxonomies most associated with the treatment groups and changes from pre-treatment to post-treatment.

4.2.3 Productivity parameters

Daily live weight gain (DLWG) was calculated as the slope between the weight of the animal at each of the weekly weighing points and the corresponding day of the trial. Using the information from dry matter intake and DLWG, feed conversion ratio (FCR) was calculated as the ratio between the average dry matter intake and DLWG for the given period. These values were calculated for each experimental period and as a cumulative for the whole experiment.

4.2.4 Methane emissions methods

After the end of the recovery period, a small cohort of animals from the STRESS group (n=6) and Control group (n=6) was selected to assess methane emissions. These animals were selected and balanced by breed, treatment, body weight and average feed intake during the last 7 weeks of the Recovery period. The methods used for measuring methane emissions follow those used by Rooke et al. (2014) in the same SRUC GreenCow facilities at Easter Howgate Farm.

Six indirect open-circuit respiration chambers were used for this experiment (No Pollution Industrial Systems Ltd., Edinburgh, UK). The total chamber volume (76 m^3) was ventilated by 4 recirculating fans set at 450 l/s. Air was removed from the chambers by exhaust fans set at 50 l/s giving approximately 2.5 air changes/h. Temperature and relative humidity were set at 15°C and 60% relative humidity, respectively. Total airflow was measured by in-line hot wire anemometers which were validated by daily measurements made with an externally calibrated anemometer (Testo 417, Testo Ltd, Alton, Hampshire, UK). Temperature and humidity were measured using sensor probes in the exhaust air outlet (Johnson Controls, Milan, Italy) and atmospheric pressure, corrected for altitude, with a Vantage Pro2 weather station (Davis Instruments, Haywood, Ca, USA). Chambers were operated under negative pressure (50 N/m^2). Methane concentrations were measured by infrared absorption spectroscopy and H_2 by a chemical sensor (MGA3000, Analytical Development Co. Ltd., Hoddesdon, UK). This analyser was calibrated with a gas mixture of known composition. Inlet air gas concentrations were recorded every 6 min in each chamber. Before the beginning of the experiment, gas recoveries were measured by releasing CO_2 at a constant rate into each chamber. The mean recovery was 98%.

Groups of steers were moved to the building where the chambers were located. Animals were loose-housed in single pens to familiarize them with the chamber environment. Single pens were 4 x 3 m in size and of an identical design to the pens within the chambers. After 6 days, steers were then moved to the chambers and remained there for 72 h, with CH_4 and H_2 measurements recorded in the final 48 h used in the analysis. Feed was provided once daily, and weight of feed within the bins was recorded at 10 s intervals using load cells. The front doors of the chambers were briefly opened at about 08.00 h daily to remove feed bins and again to replace bins

with fresh feed at approximately 09.00 h. The pens were cleaned daily between 08.00 and 09.00 h. Exact times when doors were opened were recorded.

To minimise bias caused by the entry of air when doors were opened for feeding and, as during this period (54 SD 22.5 min) steers did not have access to feed, gas concentrations measured during this period were not used for further analysis. Instead, to minimise bias, these values were replaced by the mean value of measurements (n=10) made in the last hour before doors were opened. If a steer had consumed food during that period, mean values for the hour preceding feed consumption were used. All data, including gas concentrations, airflow, temperature, humidity, atmospheric pressure and records for feed consumption, were loaded into a database. Dry air flow was calculated and corrected to standard temperature and pressure for each record of gas concentration. Daily gas production was then calculated as the average of individual values. Grams of methane per day were corrected by the average DMI of the animal (gCH_4/kgDMI); this metric was the one used for further analysis.

4.2.5 Statistical analysis

Statistical analyses were carried out using R (v3.5.2) and Genstat 16 (VSN International Ltd., Oxford, UK). The data available for productivity, Shannon diversity and methane emissions were examined for their approximation to the normal distribution using the Anderson-Darling test. Methane emission data were used after log base ten transformations. Linear mixed models (LMM) in GenStat 16 were used to assess the contribution of breed, sire, treatment and period and interaction of period and treatment as fixed effects on performance, Shannon diversity and methane emissions as outcome variables. Pen and animal nested within pen were included as random effects. For the PLS-DA analysis, the mixOmics package in R was used.

LEfSe analyses were performed using mothur (Schloss et al., 2009) and the web interface of the Galaxy workflow framework version 1.0 (https://huttenhower.sph.harvard.edu/galaxy/). The Kendall rank correlation coefficient was used to assess correlations between methane with total archaea, archaea:bacteria ratio, archaeal family and at OTU level. In all statistical analysis, significance was assumed at $p \leq 0.05$ and statistical tendencies at $p \leq 0.1$.

4.3 Results

4.3.1 Characterisation of microbial communities

The analysis was based on 462 samples, with an average of over 18,000 reads per sample. A total of 15,416 OTU were identified and assigned to eighteen different phyla according to the SILVA 128 taxonomy reference base (Quast et al., 2012). Nine of these phyla accounted for 97.7% of the sequencing reads (see Figure 4.2): *Bacteroidetes* (39.8%), *Firmicutes* (29.2%), *Proteobacteria* (17.2%), *Euryarchaeota* (5.2%), *Spirochaetes* (1.7%), *Actinobacteria* (1%), *Patescibacteria* (0.9%), *Cyanobacteria* (0.7%) and 0.7% *Tenericutes* (0.7%), with 2.9% of prokaryotes unclassified at the phylum level.

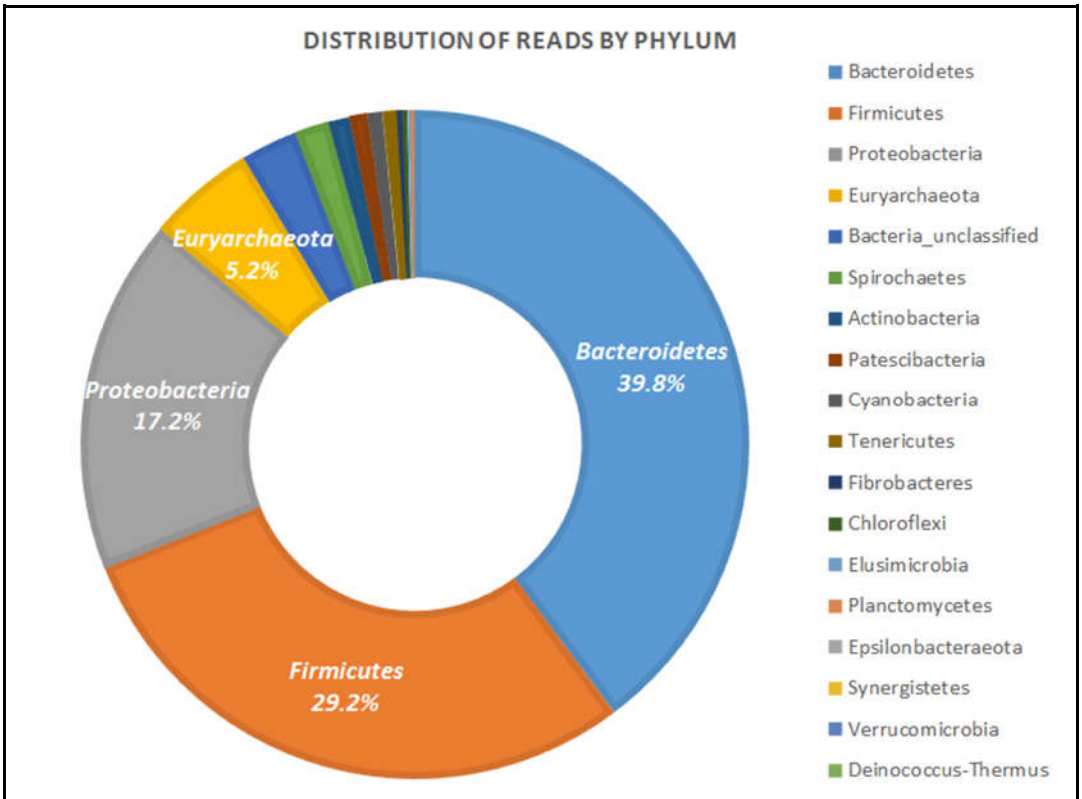


Figure 4.2. Distribution of sequencing reads at the phylum level.

There were no significant differences between the treatments or breeds on the archaea to bacteria ratio (see Figure 4.3a). However, there were significant

differences between sampling periods ($F_{4,291.8}$=2.89, p=0.023). The archaea:bacteria ratio showed a significant increase from Baseline to the end of the Recovery period, as well as significant differences between the post-ACTH timepoint and both recovery sampling points (see Figure 4.3b). No significant interaction between treatment and period was found. Sire also had a significant effect on archaea to bacteria ratio ($F_{9,295.6}$=3.31, p<0.001). Generally, there was no effect specific to the composite stressor treatment on the archaea to bacteria ratio.

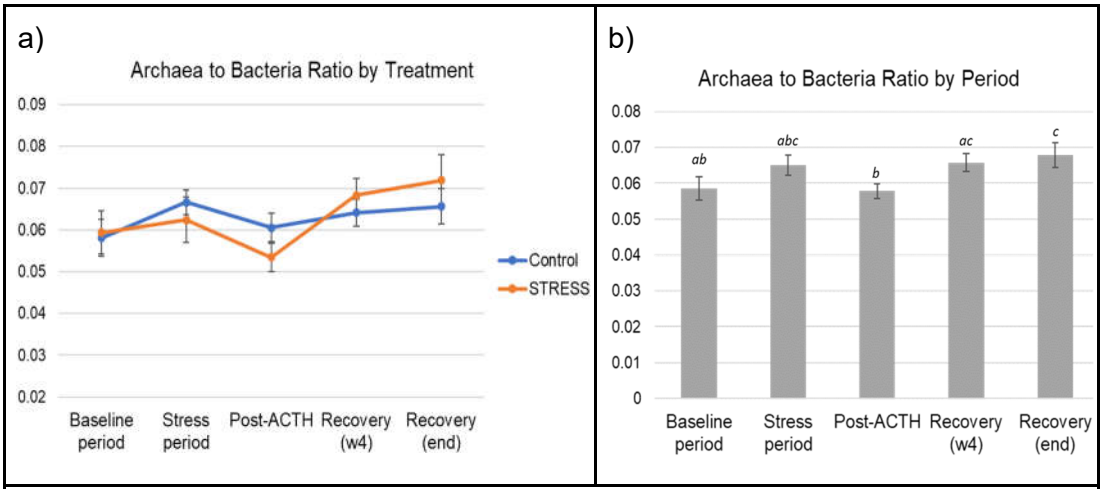


Figure 4.3. Archaea-bacteria ratio at each of the rumen sampling timepoints. Graph (a) shows no significant difference in archaea-bacteria ratio between treatment groups and (b) shows differences between timepoints. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

4.3.2 Diversity analysis

In order to evaluate alpha diversity and evenness of microbial communities, the Shannon index was calculated for all samples. A LMM was run on the Shannon index as a repeated measure to assess the average microbial diversity between treatments and over the different periods of the experiment. This analysis found a significant effect of treatment ($F_{1, 290}$=4.37, p=0.038), where control animals showed a higher diversity (4.62 vs 4.56 SED 0.031). There were also significant differences between timepoints ($F_{4, 290}$=5.49, p=<0.001) with Baseline and Stress periods being

significantly lower in comparison to Post-ACTH and both recovery timepoints (see Figure 4.4), showing a general trend for diversity to increase later in the trial.

There was a significant interaction between treatment and timepoint ($F_{4, 290}$=3.35, p=0.011). This was mainly explained by the Post-ACTH timepoint, which was the only sampling timepoint that showed a significant difference between STRESS and control animals (see Figure 4.5). More specifically, the control group showed a significant increase in diversity at the post-ACTH timepoint compared to Baseline and Stress periods, whereas the STRESS group post-ACTH diversity remained similar to that during the Baseline and Stress periods. Nonetheless, diversity in the STRESS animals significantly increased at the recovery timepoints, compared to the post-ACTH timepoint, and once again showed a similar Shannon index to control animals. Additionally, the Shannon index was affected by sire ($F_{8, 290}$=2.53, p=0.011) and there was a statistical tendency for a difference between breeds ($F_{1, 290}$=3.11, p=0.079) with AAx showing a higher Shannon index than LIMx animals (4.62 vs 4.57 SED 0.069).

Given these results, it appears that the composite stressor treatment did not significantly affect rumen microbial diversity as judged by the lack of differences between treatments by the end of the Stress period. Nonetheless, there was an apparent increase in diversity by the control group at the post-ACTH timepoint.

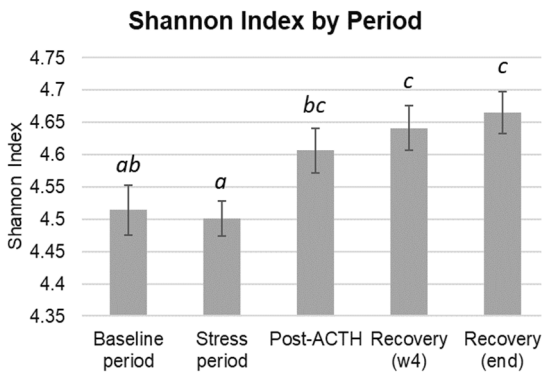


Figure 4.4. Shannon Index at each of the rumen sampling timepoints. Shannon index generally increased over time. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

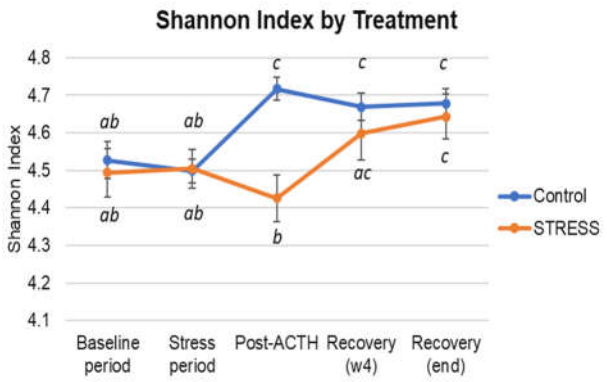


Figure 4.5. Shannon Index at each of the rumen sampling timepoints plotted by treatment. Shannon index showed an interaction between treatment and timepoint due to an increase in the control group at the post-ACTH timepoint. Values represent mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05).

The increase in alpha diversity in the control animals after ACTH treatment was analysed further. Given that the ACTH treatment was used to probe changes in adrenal sensitivity, almost all the STRESS animals (n=22) were injected with ACTH. However, only a sample of the animals in the control group (n=19) took part in the ACTH challenge allowing a comparison between STRESS animals treated with ACTH, control animals treated with ACTH and control animals that did not receive ACTH. We also evaluated any pen effects and those of sampling day since animals in different pens were treated with ACTH on different days (see Chapter 3). This analysis showed that there was no effect of ACTH administration on animals in the Control group (see Figure 4.6) and hence controls were generally different from STRESS animals independent of ACTH administration ($F_{2,\ 54}$=18.9, p<0.001). Significant pen effects were found on alpha diversity at the ACTH time point which contributed to differences between the treatments (see Figure 4.7). In particular, two pens (pen 2 and pen 7) that were sampled on the same day showed the greatest contrast in diversity.

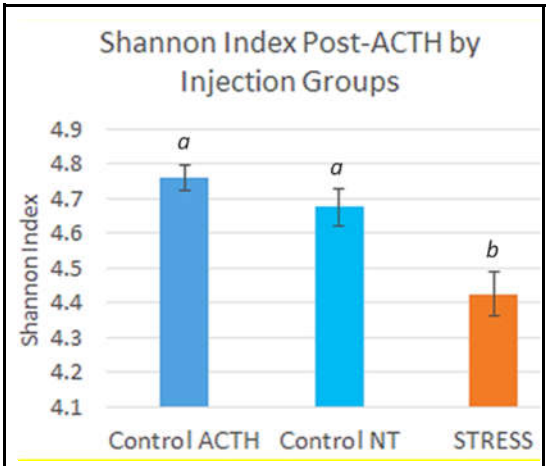


Figure 4.6. Shannon index at the Post-ACTH timepoint by injection groups. Control animals are categorised according to whether they were treated with ACTH (Control ACTH) or were not treated with ACTH (Control NT). There was no significant difference between treated and untreated controls and only between STRESS and Control groups in general. Means with different letters (a,b) are significantly different (p<0.05).

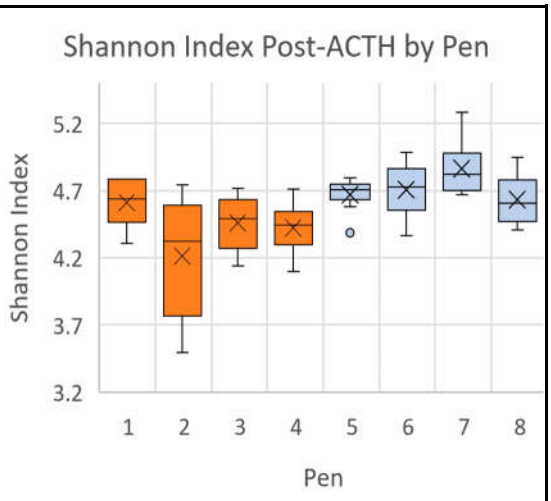


Figure 4.7. Shannon index at the Post-ACTH timepoint by pen. The boxplot shows the distribution of the Shannon index. Orange pens belong to the STRESS treatment, and blue pens correspond to the Control treatment.

Beta diversity was assessed using non-linear multidimensional scaling (NMDS) plots created from the Bray Curtis dissimilarity matrix. NMDS allowed inspection of dissimilarities based on sampling timepoints and treatments. Since the Stress period sampling timepoint occurred immediately after the STRESS animals had been continuously subjected to the treatment for 8 weeks, this was assumed to be the most critical sample with which to assess changes due to the composite stressor treatment. The NMDS plot comparing dissimilarity between STRESS and control animals at the end of the Stress period showed no clear separation or clustering due to the treatment group (see Figure 4.8). Similarly, the NMDS plot comparing the Baseline and Stress period of the STRESS treatment group did not evidence any clear separation between these timepoints (see Figure 4.9). Therefore, it appears that there were no major changes in rumen microbiome diversity due to the composite stressor treatment.

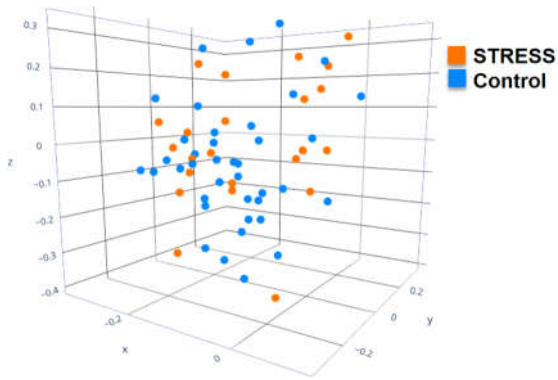


Figure 4.8. NMDS plot of Bray-Curtis dissimilarity of STRESS and control animals showing no clear separation according to treatment at the end of the Stress period.

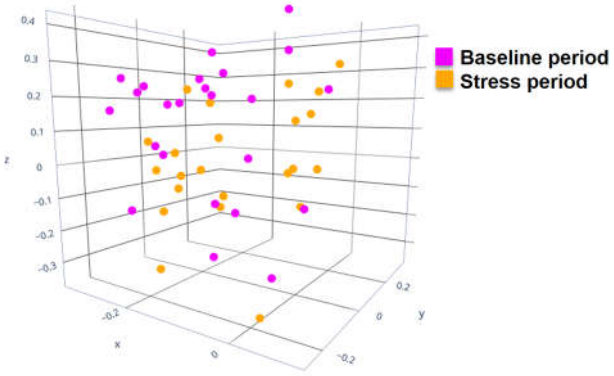


Figure 4.9. NMDS plot of Bray-Curtis dissimilarity of STRESS animals showing no clear separation between Baseline and Stress periods.

The Partial Least Squares Discriminant Analysis (PLS-DA) regression to discriminate between STRESS and Control treatments at the end of the Stress period using the OTUs as the predictor variables was an accurate model to separate the treatment categories (R^2=0.967). This model returned 49 OTUs with a VIP greater than 1. Linear Models run on these resulting OTUs and corrected using a Bonferroni correction, found only one OTU corresponding to the Prevotellaceae family (OTU21) that showed a significant increase in the STRESS group ($p<0.05$). Taking a different approach, Linear Discriminant Analysis (LDA) effect size (LEfSe) was used to identify OTUs in the STRESS group that differed significantly in abundance in the Stress period in comparison to the Baseline period. Only those OTUs with an effect size greater than 3 were evaluated. Three discriminant OTUs showed the highest dissimilarity between the Stress versus Baseline timepoint with an LDA effect size score >4. These were a bacterium of the family *Prevotellaceae* (OTU7) which was more abundant during the Stress period and two further from the class *Gammaproteobacteria* (OTU1 and OTU2) which were more abundant in the Baseline period. However, the decrease in OTU1 between Baseline and Stress periods was not significantly different between treatments. Table 4.10 shows those OTU's that had a significant change between

Baseline and Stress periods in the STRESS group with an LDA effect size of greater than 3, and that also showed a significant difference between treatments at the end of the Stress period. Although differences are present in many cases between STRESS and Control treatments, the OTUs followed a similar pattern of increase and decrease over time, suggesting that the changes were not specifically a result of exposure to stress

Table 4.10. Relative abundance of OTUs that differed significantly between Baseline (B.P.) and Stress (S.P.) periods for the treatment (STRESS) group (LDA effect size over 3). Only OTUs that differed significantly between STRESS and Control treatments at the end of the Stress period are shown. Lowest taxum level describes lowest level at which over 99% identification confidence was reported for the given OTU.

Silva 128 taxonony		**OTU**	**Relative abundance**			
Phylum	**Lowest taxum level**		**STRESS**		**Control**	
			B.P.	**S.P.**	**B.P.**	**S.P.**
Bacteroidetes	Prevotellaceae (family)	OTU00007	2.39%	4.60%	1.81%	2.37%
	Prevotellaceae (family)	OTU00021	0.15%	1.68%	0.19%	0.87%
	Prevotella (genus)	OTU00020	1.13%	1.56%	0.65%	0.94%
	Prevotellaceae (family)	OTU00131	0.15%	0.04%	0.11%	0.14%
Firmicutes	Veillonellaceae (family)	OTU00121	0.09%	0.24%	0.08%	0.11%
	Firmicutes (phylum)	OTU00022	0.64%	0.91%	0.88%	1.40%
	Pseudobutyrivibrio (genus)	OTU00103	0.18%	0.15%	0.08%	0.09%
	Lachnospiraceae (family)	OTU00118	0.12%	0.09%	0.08%	0.06%
Proteobacteria	Proteobacteria (phylum)	OTU00025	1.21%	0.56%	1.60%	1.12%
	Proteobacteria (phylum)	OTU00002	6.63%	4.32%	8.46%	6.17%
Fibrobacteres	Fibrobacter (genus)	OTU00111	0.01%	0.03%	0.07%	0.06%
Euryarchaeota	Methanobrevibacter (genus)	OTU00018	1.63%	0.68%	1.88%	1.08%

4.3.3 Productivity

There were no differences between breeds or treatments in daily live weight gain (DLWG) by the end of the trial. In contrast, there was a significant interaction between treatment and period ($F_{5,171}$=4.11, p<0.001) mainly due to differences in DLWG at the start of the trial. However, there was no difference between the treatments during the Stress and Recovery periods (see Figure 4.11). There was a significant change over time for both treatments, whereby STRESS animals showed a decrease in DLWG from Baseline to Recovery (1.55 vs 1.26 SED 0.107) whereas Control animals increased DLWG from Baseline to Recovery (1.26 vs 1.40 SED 0.070). There was also a significant effect of sire on DLWG ($F_{9,171}$=4.28, p<0.001).

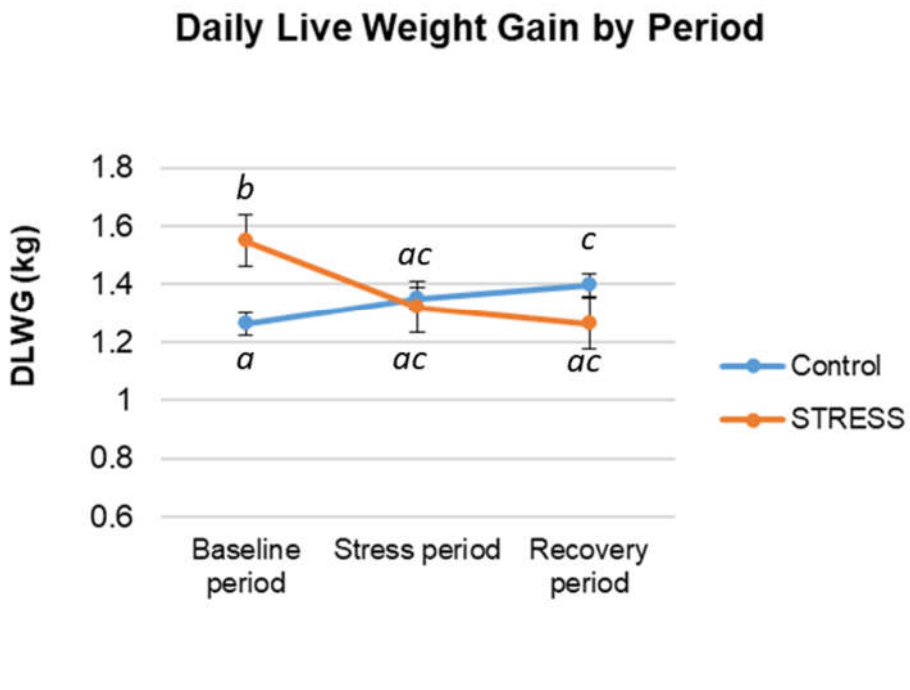


Figure 4.11. Average daily live weight gain (DLWG) by experimental period and treatment group. Means with different letters (a,b,c) are significantly different (p<0.05).

In the case of feed conversion ratio (FCR), there were no differences between breeds or treatments by the end of the trial. There were only statistical tendencies for effects of sire ($F_{9,171}$=1.81, p=0.059) and the interaction between treatment and time period ($F_{9,171}$=1.92, p=0.093), where FCR in the stress group showed a tendency to increase over time (see Figure 4.12)

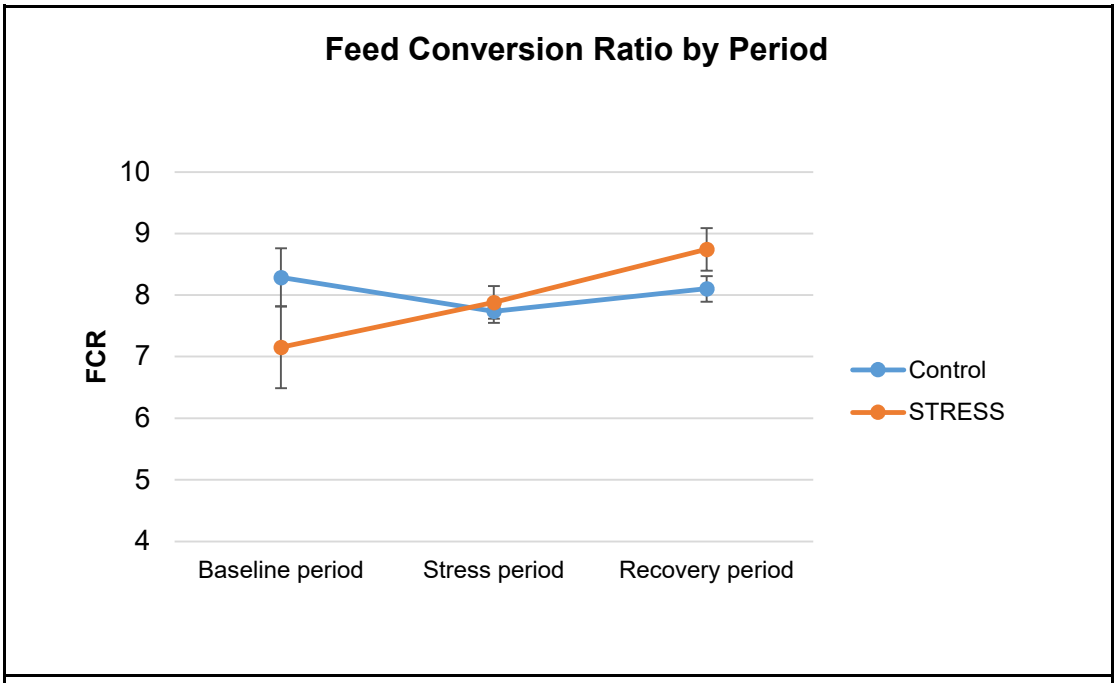


Figure 4.12. Average feed conversion ratio (FCR) by periods of the experiment and by treatment groups. FCR is presented as kg DMI/ kg LWG. There was only a statistical tendency for an interaction between time period and treatment. Values represent mean ± SEM.

4.3.4 Methane emissions analysis

Methane emissions from the small pilot study were summarised as grams per kilogram of dry matter intake (CH_4 g /kg DMI). No differences in methane emissions between treatments or breeds were found (see Figure 4.13). In this small dataset, Shannon diversity was not a predictor of individual methane emissions. Correlations

using the Kendall rank correlation coefficient did not identify any significant association between methane emissions and the archaea to bacteria ratio. There were no correlations between methane emissions and archaea relative abundance at the family level. However, analysis at the OTU level found a positive correlation (r_T 0.4654, $p<0.05$) between methane emissions and relative abundance of the genus *Methanobrevibacter* (OTU 551), as well as a statistical tendency for a positive correlation (r_T 0.537 $p=0.072$) between methane emissions and an uncultured genus of the *Methanomethylophilaceae family* (OTU481). Methane emissions positively correlated with the relative abundance of bacterial OTUs present at over 0.1% are presented in Table 4.14. Regarding the OTUs found to be correlated with methane emissions, no differences between the treatment groups or periods were found in their abundance, except for an OTU of the family *Prevotellaceae* (OTU 7) which was significantly higher in the STRESS group.

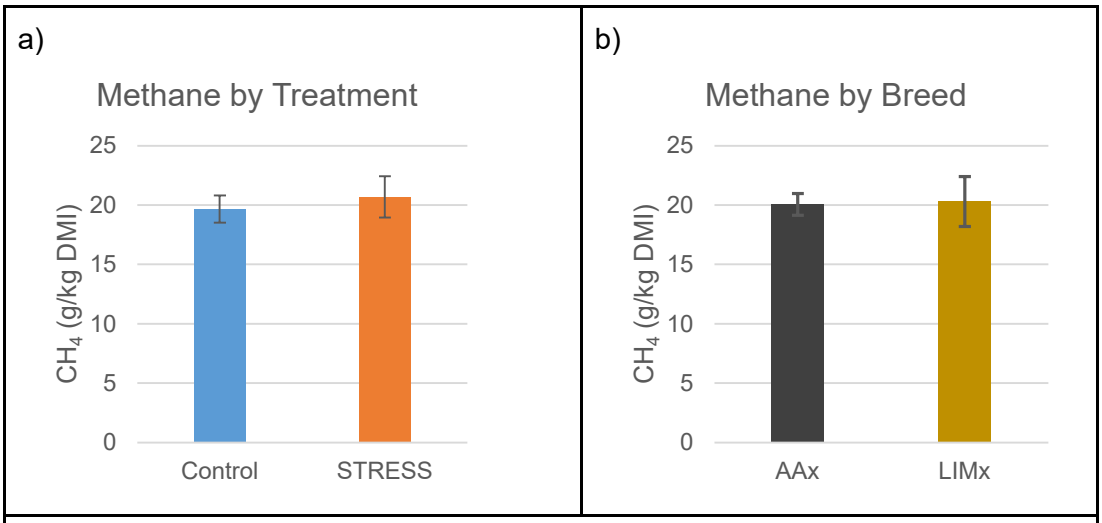


Figure 4.13. Methane emissions from a subsample of STRESS and Control animals. There was no difference in methane emissions according to treatment group (a) or breed (b).

Table 4.14. Bacterial OTUs present at over 0.1% of relative abundance and that were correlated with methane production.

Phylum	Silva 128 taxonony Lowest taxum level	OTU	Kendall τ	p-value	Mean abundance
Bacteroidetes	Prevotellaceae (family)	OTU 91	0.485	0.011	0.18%
	Prevotellaceae (family)	OTU 7	0.424	0.022	4.46%
	Prevotellaceae (family)	OTU 34	0.364	0.043	0.94%
		OTU 40	0.394	0.031	0.79%
		OTU 46	0.394	0.031	0.77%
	Rikenellaceae (family)	OTU 15	0.303	0.076	1.45%
		OTU 128	0.273	0.099	0.18%
Firmicutes		OTU 85	0.424	0.022	0.22%
	Selenomonadales (order)	OTU 87	0.364	0.043	0.24%
	Ruminococcaceae (family)	OTU 130	0.273	0.099	0.19%
Elusimicrobia	Elusimicrobium (genus)	OTU 98	0.636	0.001	0.15%

4.4 Discussion

Although changes were detected in some microbial genera over time throughout the experiment, there were no major changes directly associated with the composite stressor treatment in the rumen archaeal and bacterial populations, or in microbial diversity. Additionally, by the end of the experiment, there was no effect of stress on growth performance. The evaluation of the pilot study on the small cohort of animals to assess methane emissions found no differences between the treatment groups.

4.4.1 The effects of stress on the rumen microbiome

There were no significant differences between treatments in Shannon diversity when sampled at the end of the Stress period. Given these results, it appears that the composite stressor treatment did not affect Shannon diversity. However, Shannon diversity differences between treatments were found at the post-ACTH timepoint. The separate analysis to specifically assess changes at the post-ACTH timepoint found that ACTH treated and untreated animals in the Control group had similar Shannon diversity. Therefore, the treatment difference found at this timepoint was a general difference between STRESS and Control animals irrespective of ACTH administration.

The post-ACTH sampling occurred during the afternoon on the day after the ACTH test, i.e. once a pen was treated with ACTH, the following day in the afternoon, animals in that pen were sampled for rumen contents. The post-ACTH rumen sample was the only one collected in the afternoon due to the logistical constraint of having to perform the ACTH test in the morning for two pens; hence, the animals that had received the ACTH test the day before were sampled in the afternoon. Differences in composition between morning and afternoon samples, due for example to differences in feed and water intake across the day, may have contributed to the difference

between treatments in post-ACTH diversity. If true, this highlights the effect of sampling time as a factor affecting longitudinal microbiome analysis. For example, Welkie et al. (2010) reported that dairy cows fed a mixed forage/concentrate diet and rumen sampled at short intervals (pre-feeding, 2, 4, 6, 9 and 12 hours post-feeding) over two days, showed changes in bacterial community composition between sampling points; an effect that was more marked in communities associated with the liquid fraction of the samples.

Similarly, Shaani et al. (2018) assessed diurnal changes in the rumen microbiome and showed that metabolites produced by the rumen microbiome modify niche environments and lead to dramatic changes through the day in community composition and function, reporting up to 3 to 5-fold changes in the relative abundance of multiple taxa, independent of individual host variation and diet. Consequently, comparison of the post-ACTH timepoint with other sampling times could be confounded with diurnal changes of microbial communities as well as variation in other elements such as feeding patterns. If this is the case, then apparent differences between treatments may result simply from morning to afternoon variation being more pronounced in one treatment in comparison to the other, without this necessarily having any relationship to ACTH administration.

The NMDS plot comparing dissimilarity between STRESS and Control animals at the end of the Stress period did not show any clear separation or clustering due to the treatment group. Likewise, the NMDS plot comparing the Baseline and Stress period of the STRESS treatment group did not evidence any clear separation between the timepoints. Therefore, there do not appear to be any major changes in rumen microbiome diversity due to the composite stressor treatment.

The Partial Least Squares Discriminant Analysis (PLS-DA) regression carried out at OTU level returned a large number of OTUs predicting differences between STRESS and Control groups at the end of the Stress period. However, direct comparison of abundance of these OTUs between treatments using a Bonferroni correction found only a bacterium of the *Prevotellaceae* family (OTU 21) that showed a significant increase in the STRESS group during the stress phase. This was also one of the *Prevotellaceae* OTUs that showed a change in the Linear discriminant analysis (LDA) effect size (LEfSe) analysis. Although clearly there were changes in these OTUs, it is interesting to note that based on relative abundances, both control and STRESS treatments tended to follow the same change in direction with slight differences in magnitude. This indicates that similar changes also happened in the microbiome of the control group. Therefore, significant changes found at the individual OTU level were not different enough to affect diversity metrics or create evidently different microbial profiles between groups.

At the macro level, there is evidence that microbial diversity will remain fairly stable once the rumen has adapted to a diet (Snelling et al., 2019). However, there might still be smaller changes in abundance of individual species in response to short and longer-term factors. Some authors propose that there is a core microbiome in the bovine rumen, and although taxa may vary, given the functional requirement imposed by the rumen as an environment, selection operates for species that tend to be phylogenetically related or share similar genetic features (Jami and Mizrahi, 2012). In agreement, Taxis et al. (2015) argue that these changes in microbial communities tend to happen within the confines of maintaining a microbial network that can perform the same metabolic functions and produce the same outputs from common inputs. In essence, rather than being exclusive, rumen microbial interactions seem to occur on a functional basis, keeping a level of redundancy or overlap of function among multiple

species in order to provide the digestive function for the host and adapt to changes in feeding or environmental conditions (Henderson et al., 2015; Weimer, 2015). Therefore, it is reasonable to expect small dynamic changes in abundance at the individual species level as multiple taxa can fulfil the same metabolic function.

Taken together, the results from this experiment suggest that the stress experienced by the animals was not enough to alter the rumen microbiome. However, it is also likely that the rumen microbiome of beef cattle is quite resilient to stress. It has been shown that rumen microbiomes are able to resist and recover from perturbations, showing a remarkable general stability in order to maintain digestive function for the host (Weimer, 2015). For example, Li et al. (2012) showed that in dairy cows, a continuous 168-hour disturbance to volatile fatty acids by butyrate perturbation results in significant changes in abundance of 4 of the 5 most abundant phyla; however, these return to pre-disturbance levels by 7 days. The rumen microbiome of beef cattle has also been reported to recover within 1 week from a diet-induced acidosis challenge (Petri et al., 2013). Similarly, Deng et al. (2017) determined that transport stress did alter the rumen microbiome, linked as well to a pH drop seen post-transport, but that these changes began to revert 3 days later and the microbiome was mostly restored by day 13. More extreme changes to the microbiome such as near-total exchange of rumen contents have also rendered similar results, with the microbiome returning over time to a similar community to pre-exchange conditions (Weimer et al., 2010; Weimer, 2015; Zhou et al., 2018), confirming the resilient nature of the rumen microbiome. Therefore, even if there were any effects on diversity due to the repeated stressors, this was likely short-lived due to the resilience of the rumen microbiome. In such a case, any changes would only be detected by sampling the rumen contents within the first few days of the imposition of the composite stressor.

Individual differences in stress responsiveness could play a role in the extent to which chronic stress responses impact the microbiome, and these might not be detectable through treatment level comparisons. Further work could compare stress effects on the microbiome of animals that represent opposite ends of the distribution of stress responsiveness. Similarly, further research is necessary to investigate effects of repeated stressors on the microbiome at other sites of the GI tract of beef cattle, and also to confirm if more extreme non-commercially relevant stressors render similar results to this experiment.

4.4.2 Effects of the composite stressor treatment on productivity

There were no significant differences between treatments in daily live weight gain (DLWG) or feed conversion rate (FCR) by the end of the trial. However, STRESS treatment animals did show a significant reduction in DLWG when comparing the Baseline to the Recovery period, and also a statistical tendency for FCR to increase over this time. This could be interpreted as a reduction in growth and feed efficiency in the STRESS group as a consequence of the composite stressor treatment. However, these results are difficult to interpret as both treatments showed similar FCR and DLWG in the Stress and Recovery periods. Calculating performance parameters over short periods of time can be inaccurate in ruminants. Therefore, it is possible that the Baseline period of only 4 weeks duration could have weakened the reliability of the Baseline performance parameters, which was the only period where there were differences between treatment groups. Similarly, the smaller size of the STRESS group makes it more susceptible to other artefacts. For example, if some animals grew faster in this group (showing a higher Baseline growth rate), these animals may have reached a plateau in their growth rate earlier leading to a perceived reduction in growth rate and the accompanying increase in FCR during later stages. Therefore,

attributing this difference in performance trends between the groups to the stressor treatment may not be appropriate.

4.4.3 Relationship between stress, methane and the microbiome

Analysis of the archaea to bacteria ratio did not show significant differences between the treatment groups or breeds. Therefore, there does not appear to be an effect specific to the composite stressor treatment on the archaea to bacteria ratio. In the pilot study using the respiration chambers, methane emissions were also unaffected by treatment or breed. Interestingly, there were no significant correlations between methane emissions and the archaea to bacteria ratio. Although some authors have found such a relationship in cattle (Wallace et al., 2015a, 2015b), others have not found a relationship between methane and total archaea abundance, or archaea to bacteria ratio in cattle (Zhou et al., 2011; Danielsson et al., 2017; Tapio et al., 2017) or sheep (Morgavi et al., 2012; Kittelmann et al., 2014; Shi et al., 2014). In this regard, Shi et al. (2014) found that methane did not correlate with archaeal abundance but did correlate with archaeal gene expression. Therefore, several authors argue that methanogen abundance does not always translate directly to methane production (Hook et al., 2009; Popova et al., 2011; Schären et al., 2018) and the total archaea population might be less important than its activity (Tapio et al., 2017).

In line with the thought that specific methanogens might be more relevant than others, we did find some positive correlations between methane and specific OTUs of the genus *Methanobrevibacter* (OTU 551), as well as a statistical tendency for a positive correlation between methane and an uncultured genus of the *Methanomethylophilaceae family* (OTU481). In the past, other authors have also found a positive correlation between the relative abundance of *Methanobrevibacter species* and methane emissions (Zhou et al., 2011; Danielsson

et al., 2012; Shi et al., 2014). This has been attributed to this group having a higher affinity for H_2, allowing it to turn this molecule into methane at higher concentrations (Tapio et al., 2017). *Methanomethylophilaceae* is a member of the *Methanomassiliicoccales* order (sometimes referred to as *Methanoplasmatales* or rumen cluster C) which are commonly found H_2 dependent methanogens (Janssen and Kirs, 2008; Paul et al., 2012). *Methanomassiliicoccales* tend to be highly prevalent in animals that also have a high representation of *Methanobrevibacter* (St-Pierre et al., 2015). This methanogenic group also needs to rely on external sources of hydrogen, in this case, dependant on the reduction of methylated compounds to produce methane (Borrel et al., 2014; Brugère et al., 2014; Lang et al., 2015). The methanogen reliance on external sources of hydrogen is a major factor in determining archaeal community composition which in turn is dependent on bacterial, and also presumably protozoal and fungal communities, producing this molecule (Janssen, 2010; Kittelmann et al., 2014). This is in part the reason methanogen abundance alone does not always explain methane production directly, since relationships between host genetics, microbiome biochemical pathways and other microbial communities providing the necessary inputs for methanogenesis also contribute to determining methane emissions (Zhou et al., 2010, 2009; Roehe et al., 2016; Malmuthuge and Guan, 2017; Schären et al., 2018). This notion is reinforced in a recent study using comprehensive network analysis on relative abundances of all ruminal microbial genera (archaea, bacteria, fungi, and protists) and their genes (Martínez-Álvaro et al., 2020). That study suggests that rumen methane emissions are indeed mainly explained by non-archaea microbial communities and their metabolic pathways, rather than only being methanogen-driven.

The phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* have consistently been found to be the most abundant in rumen microbial communities (Bekele et al., 2010;

Henderson et al., 2015; Weimer, 2015). In general, the *Prevotellaceae* family is highly represented with *Prevotella* typically being the main bacterial genus and present in a large number and variety of species (Stevenson and Weimer, 2007; Kim et al., 2011). Previous results have found some OTUs of the genus *Prevotella* that are positively correlated with methane production, whereas other work suggests that a positive correlation depends upon how relevant the species is in regard to H_2 production or utilization. For example, a study by Danielsson et al. (2017) demonstrated that the presence of some *Prevotella* OTUs was positively correlated with methane, whereas others of the same genus was negatively correlated. *Prevotella* species can have proteolytic activity and produce a variety of extracellular degradative enzymes to decompose a variety of substrates with considerable variation between species (Stevenson and Weimer, 2007). However, as a large proportion of rumen-associated *Prevotella* are still uncultured, their individual role is difficult to determine (Bekele et al., 2010). Therefore, the association of species of *Prevotella* with methane could have many explanations, including being a niche marker, producing compounds promoting methanogenesis or as net hydrogen utilizers. For example, Denman et al. (2015) found that an additive that reduced methanogenic archaea increased the abundance of *Prevotella* species that utilise H_2. In addition to differences in methane emissions, *Prevotella* changes have also been associated with feed efficiency in beef cattle (Myer et al., 2015). Therefore, further research to understand the role of *Prevotella* species using recently identified rumen metagenome-assembled genomes (Stewart et al., 2019) on ruminal fermentation and methanogenesis would be of benefit.

Similar to *Prevotella*, *Selenomonadales* are another H_2 utilizing group, whereas fibrolytic bacteria, such as cellulolytic *Ruminococcaceae* are well studied H_2 producers (Denman et al., 2015; Farghaly et al., 2019). *Elusimicrobia* is a rather

recently identified bacterial phylum, with the first sequences identified from termite-gut samples (Ohkuma and Kudo, 1996). Since then, it has also been identified in other environments such as soil, aquatic environments, the gut of some animals and in the rumen (Méheust et al., 2019). This group is thought to ferment and deliver hydrogen to other microorganisms and includes species linked to the biosynthesis of a cofactor involved in catalysing methane release in the final step of methanogenesis (López-Archilla et al., 2007; Zheng et al., 2016; Moore et al., 2017; Méheust et al., 2019). This could explain why this group was correlated with methane emissions in our study, although no literature has previously reported this association. Further taxonomic identification of bacterial OTUs that correlated in this study to methane emissions would be interesting in order to understand further the interaction of these groups with methane.

4.5 Conclusion

In conclusion, although changes were detected in some microbial genera over time throughout the experiment, the composite stressor regime applied did not cause substantial changes in rumen microbial diversity or methanogenic archaea populations. Additionally, by the end of the experiment, there were no differences between treatment groups in growth performance, feed efficiency or methane emissions. These results suggest that the rumen microbiome of beef cattle might be resilient to repeated mild stressors that do not interfere with normal feed intake. This chapter was a first step towards enhancing our understanding of the dynamics of the rumen microbiome in response to repeated stressors. Further research needs to examine more closely the links between biological changes in response to severe chronic stress and microbiota resilience in the rumen and other sites of the GI tract.

Chapter 5 - General discussion

5.1 Summary of main findings

The overall aim of this thesis was to understand how commercially relevant stressors affect ruminal microbial populations, as well as concurrent effects on behaviour, feed efficiency and methane emissions in beef cattle. The main findings of both experiments were the following:

Chapter 2

- Treatment with the exogenous glucocorticoid dexamethasone induced transient changes in behaviour and physiology, such as changes in activity, and faecal cortisol, in beef cattle.
- Dexamethasone did not induce any significant and general changes in the rumen archaea population or microbial communities.
- There appears to be a lack of direct effect of exogenous glucocorticoids in producing changes in ruminal microbial populations.

Chapter 3

- Cortisol responses, although different between control and stress treatment groups, could not be specifically attributed to the composite stressor treatment.
- The ACTH challenge employed did not detect any significant differences in adrenal sensitivity between treatments.
- There were no effects of treatment on feed intake, agonistic or affiliative behaviour.

- The composite stressor treatment resulted in differences between treatments in some parameters of activity and an attention bias test.
- The results indicate that beef cattle may show some resilience to repeated but predictable stressors. However, not all animals experience stressors in the same way, and likely there was a degree of variability in coping strategies and level of resilience.

Chapter 4

- Although some longitudinal changes were detected in some microbial genera, the composite stressor treatment applied did not cause substantial changes in rumen microbial diversity or methanogenic archaea populations.
- The pilot study did not show differences in methane emissions between the treatments.
- There were no differences between treatment groups in growth performance or feed efficiency.
- Results suggest that the rumen microbiome of beef cattle might be resilient to repeated mild stressors that do not interfere with the normal feed intake.

Taken together, the results from this thesis suggest that there is a resilience of the rumen microbiome, including with respect to methanogen populations, to high levels of exogenous glucocorticoids administered over 3 days, and to a composite stressor applied over 8 weeks. Similarly, there appears to be a resilience of the host to the composite stressor regime applied here with respect to their behaviour and HPA response.

From a more mechanistic perspective, the use of dexamethasone was an interesting option to assess the effects of a circulating glucocorticoid in the rumen microbiome in

a controlled manner. In this experiment, monitoring faecal cortisol metabolites did serve to confirm that, as was expected, in response to dexamethasone the HPA axis responded with a sustained negative feedback on cortisol production, corresponding to the effect expected from high levels of circulating endogenous cortisol. However, no changes were detected in the rumen microbiome on the day of sampling, suggesting a lack of direct effect of glucocorticoids and showing the resilience of the rumen microbiome to this compound. However, this model has its pitfalls as dexamethasone will not have the same physiological and central effects as exposure to actual real-life stressors. Therefore, these results could not be directly extended to infer whether stress will produce changes in the microbiome. Similarly, dexamethasone-treated animals did show some predicted effects on behaviour, which also served to confirm the impact of dexamethasone on normal biology. Nonetheless, if an effect of dexamethasone had been found on the microbiome, we would not have been able to separate the effects of dexamethasone from the effects of the behavioural changes which might have impacted the rumen environment through changes in feed and water intake. This also serves to show how challenging it is to stimulate a physiological response to stress separate from a behavioural response.

To complement the first experiment and to study the effects of real-life repeated stressors on the microbiome, the second experiment used a composite stressor treatment. Although none of the four commercially relevant stressors was extreme on its own, it was expected that the mixture of stressors would combine to create a chronic intermittent stressor (Ladewig, 2000). As described in the results of Chapter 3, the changes in behaviour were minimal and no changes over time were found in HPA axis stress responses that could be attributed to the stress regime, perhaps due to habituation to the stressors.

The results from Chapter 4 found that the stressor regime applied did not cause substantial changes in rumen microbial diversity or methanogenic archaea populations. Given that Chapter 3 found little evidence that the cattle were in a state of chronic stress, the resilience of the rumen to the stress regime was not surprising. The focus of the second experiment was on using multiple stressors that would not directly affect feed and water intake. However, perhaps stressors that do interfere with normal water and feed intake (e.g. competition for feed) could have a more substantial and more direct effect on the rumen microbiome. In this sense, previous studies looking at heat stress (Uyeno et al., 2010; Chen et al., 2018; Baek et al., 2020) and acute changes due to transport (Deng et al., 2017; Li et al., 2019) did find more overt changes in the microbiome, possibly due to interference with normal feeding patterns.

Perhaps due to its critical function for the host, the ability to adapt the rumen microbiome to external events such as a change in diet, reduced intake, or internal homeostasis (such as acidosis or dehydration) might take precedence over responding to closely repeated mild stressors that do not affect feeding patterns dramatically. Conversely, heat stress in beef cattle has been shown to reduce DMI and feed efficiency, and to curtail growth (Mader et al., 2006; Marchesini et al., 2018; Summer et al., 2019). These strong effects on feed intake and nutrition are likely coupled with changes in the supply of nutrients to the rumen; hence, they might be expected to affect the rumen microbiome directly. More research is needed to identify the relationship between stressors and their direct effect on feeding patterns, water intake and ruminal pH and how these, in turn, may affect the microbiome.

The greatest strength of this thesis is the complementarity of the experiments. This is evident in the fact that each experiment had its own set of drawbacks, and each experiment alone would have important caveats to the conclusions that could be drawn from it. For example, the dexamethasone trial answers the question about the

lack of effects of circulating glucocorticoids on the rumen microbiome; however, it misses the applicability to real-life stressors. On the other hand, although the composite stressor treatment experiment may have created acute stress in the days after its onset, by the time of rumen contents sampling at the end, there was no evidence the animals were experiencing any chronic stress, and so any changes in the microbiome due to acute stress may have reverted. However, when these experiments are combined, their findings become more robust and relevant. In this regard, the evidence that the higher glucocorticoid levels of the dexamethasone trial did not lead to overt changes in the microbiome suggests that it is unlikely that an acute response to the composite stressor treatment led to short-term changes in the microbiome that we failed to sample. At the same time, the latter experiment examines the effects of real-life stressors which was missing in the dexamethasone experiment. Therefore, this information does suggest that beef cattle show resilience to the composite stressor regime applied; not surprisingly, a resilience also shared by their rumen microbiome.

It has been previously documented that the rumen microbiome shows an substantial degree of resilience and capacity to recover from severe perturbation, even as extreme as rumen contents exchange (Weimer et al. 2010; Zhou et al., 2018), due to the critical importance of maintaining stability in digestive function for the host (Weimer, 2015). The current thesis now adds the finding that there is also a remarkable level of resilience of the rumen microbiome in response to stressors experienced by the host. Perhaps given the crucial function of the rumen microbiome, this stability and resilience in response to stress is necessary. This raises one possibility that the effects of stress on productivity found in the literature could be more related to changes in the lower gastrointestinal tract microbiota, akin to the case in

monogastrics (O'Mahony et al., 2009; Bailey et al., 2010). More research is needed to evaluate if stress may affect the microbiota of the lower GI tract in ruminants.

In neither experiment did the stressor regime applied cause substantial changes in methanogenic archaea populations. The respiration chamber pilot trial did not show differences between treatment groups after the stress period. However, this was just a small pilot, and it is not possible to draw definitive conclusions on whether stress affected methane emissions. This is because archaeal abundance is not always a good predictor of methane emissions, as discussed in Chapter 4. Secondly, methane emissions were only collected on a small cohort of animals, and could not be measured during the period when the stressors were applied, but instead more than nine weeks after the end of the composite stressor treatment. Nonetheless, the fact that productivity was not affected by the stressors used in either experiment, and given the absence of changes in the rumen microbiome, it seems unlikely that the stressors would have affected the methane output of these animals. However, future work should confirm that stress does not affect methane emissions using more timely methane emissions data, especially given the fact that any effects of stress on growth and productivity would effectively increase emission intensity per kg of meat or kg DMI.

Regarding the welfare implications of this research, it is necessary to add a note of caution before inferences or assumptions can be made. The composite stressor treatment was comprised of commercially relevant stressors but which were applied much more frequently than would occur on a commercial farm. However, given that the long-term effects of this stressor treatment on physiology were not evidenced in this experiment, there could be an erroneous temptation to assume that this kind of stress is inconsequential for the animals, and that cattle may be more resilient to repeated and predictable stressors than initially expected. This could lead to an

incorrect assumption that any farm applying these stressors very frequently would not need to worry about the welfare of their animals. However, these are not assumptions that could be taken from this study. Firstly, since the animals were weighed weekly and received much human contact, they could have become desensitised to close human contact and the handling required for most of the stressors. Furthermore, the focus of this thesis was primarily to identify physiological evidence of a stress response to relate to microbiome changes, not to examine whether the cattle's welfare, in terms of subjective experience, was altered. Assessing specific welfare-related behavioural changes would have required more extensive behavioural tests and analysis throughout the entire study. Animal welfare would also have to be considered on an individual animal basis rather than at the treatment group level in order to evaluate any ethical issues. Therefore, it is beyond the scope of the thesis to define stressor thresholds beyond which the welfare of beef cattle will be compromised.

5.2 Limitations

As described in the previous section, the animals used in the second experiment had extensive exposure to general handling, as well as with the stressors throughout the experiment. This situation may have reduced the impact these stressors had for the treatment group, as it would reduce the novelty of the handling situation and make the stressors more predictable. It is known that a reduction in novelty and increase in predictability may influence the aversiveness of a given stimulus (Koolhaas et al., 2011) which could affect the stress response; therefore, more naive animals could have shown different responses. Similarly, the animals in the control group had to undergo management procedures such as weekly weighing, accelerometer attachment, faecal sampling, blood sampling, rumen contents sampling and the ACTH challenge. Therefore, it could not be said that these animals were completely

undisturbed. Their experience at times could have been stressful and, if so, the less frequent exposure to the stressors would have limited opportunities for habituation and, concurrently, the stressors would have been less predictable. It is possible that using other more severe stressors for the treatment group would have created a more considerable distinction between the stress and control treatments and would have aided in the detection of stress-related effects on the rumen microbiome.

However, when the experiment was being designed, two essential characteristics of the treatment were that it would need to have some relevance to real-world commercial farm stressors, and it should not interfere directly with feeding, as this could have a confounding effect with any changes in the microbiome. Therefore, one strategy would have been to impose a repeated stressor at levels typically found on commercial farms. However, this would have reduced the likelihood that the regime was severe enough to show the effects of chronic stress. On the other hand, severe chronic stressors found in the literature, such as substantial restrictions on lying time (see Munksgaard et al.,1999), would likely induce a chronic stress response but would have little relevance to commercial beef farming. The approach taken was a compromise between both scenarios, applying commercially relevant stressors but at levels and frequencies exceeding what would be expected on the regular beef farm. This approach has its limitations; nonetheless, it does show that the suite of common stressors applied at levels beyond standard farm practice had no effects on the rumen microbiome. From the perspective of understanding basic biology, it would be interesting to confirm if other more severe stressors also cause no severe disruption to the rumen microbiota.

Another limitation was the difference found in baseline cortisol between the treatment groups in the second experiment, where Control animals showed higher basal cortisol compared to STRESS animals. As was described in Chapter 3, these two groups

were managed in exactly the same way up until the composite stressor treatment commenced. This situation seems to have occurred merely by chance; however, in hindsight, there are some factors that may have contributed to this pre-existing difference. The first one relates to the sample size used for statistical analysis, as the Control group was much larger (40 animals) compared to the STRESS Stay group (24 animals). Although the groups were balanced by breed, age, weight and sire, the smaller sample size in the STRESS treatment may have enabled some disparities to be created by chance, affecting the distribution of factors with potentially substantial effect, such as temperament and stress responsiveness, which were not traits that could be known in advance before the start of the trial. However, of interest is an additional analysis I performed (data not included in the thesis) where a comparison of STRESS treatment animals (n=24) matched to animals in the Control group that had similar cortisol at the Baseline sampling period (n=24), showed no differences between groups in cortisol throughout the trial. This confirms that the stress treatment did not affect the cortisol profile. However, using this analysis further would have been controversial due to the systematic exclusion of other animals based solely on a desire to enforce a comparable baseline cortisol level for the two treatments. However, statistical analysis using the entire population based on LMM with animals as their own controls still allowed us to successfully identify the lack of changes in cortisol due to treatment.

The collection of rumen fluid through nasogastric intubation allowed us to sample a larger number of animals, and reduces the welfare concern of performing more extreme procedures traditionally used in ruminant research (e.g. rumen fistulation). However, this is not free of some limitations, as it can introduce a source of variability to the samples. For example, it is only possible to collect samples from the liquid fraction of the rumen contents. Some authors argue that this could lead to differences

in microbial community structure when not including the solid fraction of the rumen contents (Li et al., 2009; Jami and Mizrahi, 2012; Jewell et al., 2015). Nonetheless, due to its less invasive nature, intubation has become a widespread alternative to surgical cannulation to obtain rumen contents samples (Henderson et al., 2013). In this regard, other authors have found that the microbiome composition of the liquid fraction of the rumen contents is still similar enough to draw general conclusions about diversity and composition (Ramos-Morales et al., 2014; Ji et al., 2017). However, the existence of this bias and limitation must be taken into consideration.

Regarding the severity of the rumen contents sampling procedure, further refinement is possible. Recent work carried out by Tapio et al. (2016) demonstrated that it is possible to collect residue from bolus contents in the mouth using swabs, and via bioinformatic filtering their metagenomic information from these samples, this can be used as a proxy of rumen microbiota. Therefore, in future studies, it would be possible to assess more animals without having to rumen contents sample, allowing more samples to be collected and reducing the severity of the procedure considerably compared to nasogastric intubation.

An important point to consider in stress and microbiome studies are the effects of intra-day variation in both endocrine responses (such as cortisol) and microbial community composition. Circadian effects in cortisol responses are a well-known phenomenon (Carnes et al., 1988; Ladewig and Smidt, 1989; Mormède et al., 2007). Similarly, slight changes in the microbial community throughout the day have now been reported (Welkie et al., 2010; Shaani et al., 2018), highlighting the importance of standardising the sampling procedure to reduce any spurious effects on longitudinal studies evaluating these parameters. In our study, this situation was taken into consideration for the design of the study. However, due to logistical constraints, the post-ACTH rumen contents sampling was done later in the day, which affected

the diversity results for the treatment groups differently. Therefore, this intra-day variation is a crucial point to take into consideration for experimental design and analysis of future projects, as well as in the critical review of published research studies.

5.3 Future work

The data obtained during this project was extensive and could still be used to analyse further research questions complementary to this thesis. For example, given the variation in individual responses, a portion of the animals might have experienced more stress than others, which could be masked when running group comparisons. As such, identifying those animals with differential responses could provide insight into those animals where welfare could have been more compromised and stress effects on the rumen more marked. Therefore, further analysis could be made at an individual animal level, as well as creating subsamples to perform extreme group analysis based on the different traits. This would allow analysis to address complementary research questions, such as the evaluation of the microbiome response of those animals with the highest and lowest stress responsiveness. Alternatively, it would be possible to identify animals with the most divergent response in their microbiomes to the imposition of stress, and to assess if any of the behavioural or physiological parameters recorded explain this divergence.

The strategy used to assess temperament from video recordings of the animals worked well and provided interesting results. If this method could be automated, it could provide a new tool to assess temperament in research without the time-consuming process of temperament testing and perhaps could have commercial applications. Future work might look into automating this process. As part of my placement in Ireland with collaborators at NSilico Cork, we worked on using Deep

Learning to analyse total distance and speed of steers during the attention bias test (this data is not part of the thesis), and this work led to a conference paper. It would be interesting to evaluate the applicability of this approach to temperament testing.

One central finding of this thesis has been the evidence of resilience of the rumen microbiome to both glucocorticoids and repeated stressors. More research is needed to understand the internal mechanisms that allow the microbiota to be able to resist and recover from perturbation. Additionally, it would be interesting to know what makes a microbiome more or less resilient, as this might be dependent on the microbiome itself or host factors that drive this resilience, or the interaction of both microbiome and host factors. Similarly, it would be of commercial relevance to explore if this resilience has any implications for the productivity and overall health of the ruminant host. Elucidating this connection between the microbial community and the individual host could provide insights into the reasons behind individual animal variation in productivity and could improve herd resource use efficiency.

Future research might utilise more extreme and less predictable stressors to answer from a purely biological perspective, how stress affects the rumen microbiome. Additionally, although glucocorticoids and repeated mild stressors do not appear to affect the rumen microbiome, further studies are necessary to investigate any effects of stress and glucocorticoids on microbial communities in other sites of the GI tract in beef cattle. Furthermore, there is a need to study the effects of stressors that directly affect feeding and drinking behaviour which likely affect rumen pH and dehydration.

More work needs to address the development of methane proxies that can be easily assessed on-farm, as well as the development of methane emissions predictions from the microbiome sequence data. Having these kinds of technique would be helpful as methane tends to be measured as a long-term trait in order to evaluate genetic effects

or those of diet. However, it is not as useful to determine the effects of a treatment real-time, for example when assessing if a stressor increases methane output. To date, we still do not know if methane output is affected while animals are under stress, since measuring methane during the application of any treatment is nearly impossible. Therefore, knowledge in this area and its applicability to assess changes in methane output during stress would be of value.

5.4 Conclusions

In conclusion, the findings of this thesis suggest that circulating glucocorticoids do not appear to affect the rumen microbiota balance directly, although it is still to be studied whether they affect microbial communities in other sites of the GI tract. Some differences in behaviour, but not cortisol, were found in response to the composite stressor treatment, suggesting that beef cattle might be resilient to repeated but predictable stressors. The stressor regime applied did not cause substantial changes in rumen microbial diversity or methanogenic archaea populations. This thesis was a first step towards enhancing our understanding of the dynamics of the rumen microbiome in response to stress. Further research needs to examine more closely the links between biological changes in response to severe chronic stress and microbiota resilience in the rumen and other sites of the GI tract.

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Appendices

Appendix 2.1 Contents of diets used in experiment results Chapter 2

Components (shown as g/kg DM)	**Concentrate Diet**	**Forage Mixed Diet**
Grass Silage	-	169
Wholecrop Barley	-	325
Barley Straw	80	-
Barley	650	272
Maize Dark Grains	239	202
Molasses	21	22
Minerals	10	10

Appendix 2.2 PBS-glycerol media composition

Components	Weight (g / litre solution)
NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4.2H_2O$	1.43 g
KH_2PO_4	0.24 g
Glycerol (87% stock)	280 ml

Volume adjusted to 1 Litre using deionized sterile water and pH adjusted to 7.4

Appendix 3.1 Contents of diet used in experiment Chapters 3 and 4

Components (g/kg DM)	
Wholecrop Barley	493
Barley	232
Maise Dark Grains	242
Molasses	24
Minerals	9